

Epithelial Activation in Chronic Obstructive Pulmonary Disease (COPD)

By

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Abstract

Background: Early on I identified that reticular basement membrane (Rbm) in current smokers with COPD was highly fragmented, with cracks termed “clefts” containing cells. This looked like the described hallmark of EMT (epithelial mesenchymal transition). I followed this preliminary observation with a comprehensive cross-sectional study in which I hypothesized that the airway epithelium is activated in smokers, and that this may promote EMT, but that this will be especially active in COPD. As a part of my thesis, I also investigated the expression and activity of the anti-inflammatory enzyme HDAC2 (histone deacetylase 2) which is reported to be reduced in COPD lungs and may account for associated pulmonary inflammation. I hypothesised that the current literature is correct in stating that HDAC2 is down-regulated in COPD airways and also that these reduced HDAC2 levels are normalised by aggressive inhaled corticosteroid (ICS) therapy and smoking cessation in patients with COPD.

Methods and results: Endobronchial biopsies (ebb) from current smokers with COPD (COPD-CS) and ex-smokers with COPD (COPD-ES), smokers with normal lung function (NS) and never-smoking controls (NC) stained for markers of EMT, matrix metalloproteinase-9 (MMP-9), fibroblast protein (S100A4), epidermal growth factor receptor (EGFR), vimentin and cytokeratins. To confirm the extent of suppressed HDAC2 ebb were immuno-stained for HDAC2. In a double-blind, randomized, placebo-controlled study, I assessed the effects of ICS on Rbm fragmentation and HDAC2. *Compared* to NC, there was significant fragmentation of the Rbm in COPD-CS, COPD-ES and NS groups. COPD-CS, NS and COPD-ES demonstrated increases in staining for: basal epithelial S100A4, epithelial EGFR and MMP-9 and S100A4 for cells in Rbm “clefts” compared to NC. Dual staining revealed that vimentin (a mesenchymal marker) co-stained with cytokeratin (an epithelial marker). ICS normalised Rbm fragmentation. Compared to NC there was significant increase in HDAC2 positive cells in NS in the lamina propria (LP) but a decrease in COPD-CS. However, this latter abnormality was due to a reduction in total LP cells and not % cell HDAC2 staining. There were significantly more

HDAC2 positive cells in COPD-ES compared to COPD-CS, but again due to an increase in total cell numbers. ICS made no difference to HDAC2 staining.

Conclusions: This is the first description of likely EMT in smoking and COPD. ICS reversed Rbm fragmentation. HDAC2 expression was reduced in smokers but confounded by changes in cellularity. Quitting does seem to have a real effect on up-regulating HDAC2, but it is not affected by ICS.

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Publications

★Data presented in these papers/abstracts are discussed in the thesis.

Manuscripts

★ **SS Sohal**, DW Reid, A Soltani, C Ward, S Weston, HK Muller, R Wood-Baker, & EH Walters. “*Reticular basement membrane fragmentation and potential epithelial mesenchymal transition (EMT) in the airways of smokers is exaggerated in COPD*”. **Respirology**, August (Vol 15, issue 6) 2010.

A Soltani, DW Reid, **SS Sohal**, R Wood-Baker, S Weston, HK Muller & EH Walters “*Basement membrane and vascular remodelling in smokers and chronic obstructive pulmonary disease: a cross-sectional study*”. **Respiratory Research**. 2010 July 30; 11(1):105.

Abstracts/Conference Presentations

★ **SS Sohal**, DW Reid, C Ward, S Weston, R Wood-Baker, EH Walters. “Evidence of epithelial-mesenchymal transition in COPD?” Oral presentation (presented by SS Sohal) published in *Respirology* as an Abstract, April 2009, 14, page-A18.

★ **SS Sohal**, DW Reid, A Soltani, C Ward, S Weston, HK Muller, R Wood-Baker, EH Walters. “Smoking has potential to initiate basement membrane disruption and epithelial mesenchymal transition in COPD”. Oral presentation (presented by SS Sohal) published in *European Respiratory Journal*, September 2009; 34: Supplement 53, 264S.

★ **SS Sohal**, DW Reid, A Soltani, C Ward, S Weston, HK Muller, R Wood-Baker, EH Walters. “Smoking has potential to initiate epithelial mesenchymal transition (EMT) in the airway mucosa”. Published in *Respirology* as an Abstract (A32), 2010, 15 (Suppl. 1), A11-A40.

★ Soltani A, **SS Sohal** Reid D, Muller HK, Weston S, Wood-Baker R, Walters EH. “Basement membrane remodelling in COPD responds inhaled corticosteroids”. Published in *Respirology* as an Abstract (A33), 2010, 15 (Suppl. 1), A11-A40.

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“Inhaled corticosteroids (ICS) in airway remodelling in COPD”. **Abstract**, Thorax
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Abberivations & Symbols

I	Roman Numeral 1
II	Roman Numeral 2
III	Roman Numeral 3
IV	Roman Numeral 4
V	Roman Numeral 5
VIII	Roman Numeral 8
α	Alpha
β	Beta
γ	Gamma
g	Grams
μ	Micron
μm	Micrometer
mm	Millimeter
<	Less than
>	Greater than
\leq	Less Than or Equal To
\geq	Greater Than or Equal To
Sqmm	Square Millimeter
α-SMA	Alpha -Smooth Muscle Actin
ATS	American Thoracic Society
APC	Adenomatosis polyposis coli
AP-1	Activator Protein-1
BMP-7	Bone Morphogenic Protein-7
BOS	Bronchiolitis Obliterans Syndrome
BAL	Bronchoalveolar Lavage
BSA	Bovine Serum Albumin
BALT	Bronchial Associated Lymphatic Tissue
bHLH	Basic Helix-Loop-Helix
CS-GCR	Corticosteroid-Glucoctricosteroid Receptor
COPD	Chronic Obstructive Pulmonary Disease

CTGF	Connective Tissue Growth Factor
CREB	Cyclic AMP Response Element Binding Protein
CBP	CREB Binding Protein
COX-2	Cyclo-oxygenase-2
CS	Corticosteroids
DNA	Deoxyribonucleic Acid
DAB	Diaminobenzidine
DeltaEF1	Delta-crystalline enhancer factor-binding factor 1
ERS	European Respiratory Society
EGFR	Epidermal Growth Factor Receptor
FEV1	Forced expiratory volume in one second
FVC	Forced Vital Capacity
EMT	Epithelial-Mesenchymal Transition
ECM	Extracellular Matrix
FGF	Fibroblast Growth Factor
FSP-1	Fibroblast Specific Protein-1
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GOLD	Global Initiative for Chronic Obstructive Pulmonary Lung Disease
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GSK-3β	Glycogen Synthase Kinase 3 Beta
GTP	Guanosine-5'-triphosphate
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GRE	Glucocorticoid Response Elements
GR	Glucocorticoid Receptor
HGF	Hepatocyte Growth Factor
H & E	Haematoxylin and Eosin
HRP	Horseradish Peroxidase
HES1	Hairy Enhancer of Split
HDAC-2	Histone deacetylase –Two
HAT	Histone Acetyltransferase
Ha	Hair Keratins
ICS	Inhaled Corticosteroids

IL	Interleukins
IPF	Idiopathic Pulmonary Fibrosis
IGF-II	Insulin Growth Factor-II
ILK	Integrin-Linked Kinase
Id	Inhibitors of Differentiation
iNOS	Inducible Nitric Oxide Synthase
IgG	Immunoglobulin G
LEF-1	Lymphoid Enhancer Factor-1
LABA	Long Acting Beta Agonist
MDCK	Madin-Darby Canine Kidney Cell Line
MET	Mesenchymal-Epithelial Transition
MAPK	Mitogen-Activated Protein (MAP) kinases
MMP	Matrix metalloproteinases
mRNA	Messenger Ribonucleic Acid
NCoR	Nuclear Receptor Co-repressor
NF-κB	Nuclear factor kappa B
NO	Nitric Oxide
O₂⁻	Superoxide Anions
OCT	Ornithine Carbamyl Transferase
PI3K	Phosphoinositide-3-Kinase
PCAF	p300/CBP-Associated Factor
PAI-1	Plasminogen Activator Inhibitor-1
PI3 kinase	Phosphatidylinositol 3-Kinase
qRT-PCR	Quantitative Reverse-Transcriptase–Polymerase-Chain-Reaction
Rbm	Reticular Basement Membrane
RNA	Ribonucleic Acid
RT	Room Temperature
SLPI	Secretory Leukocyte Protease Inhibitors
STAT-3	Signal Transducer and Activator of Transcription 3
SIP1	Smad-interacting protein-1
SERPIN2	Serine Protease Inhibitor
SMRT	Silencing Mediator of Retinoid and Thyroid Hormone Receptors
TGF-β1	Transforming growth factor-Beta1

TNF-α	Tumor Necrosis Factor-Alpha
TLE3	Transducin-Like Enhancer
TIMPs	Tissue Inhibitors of Metalloproteinases
TORCH	Toward a Revolution in COPD Health
TBP	TATA box-binding protein
UCH	Ubiquitin Carboxyl-Terminal Hydrolase
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor

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Chapter 1

Introduction and Preliminary Data

1.1 Background

Chronic obstructive pulmonary disease (COPD) is characterized by progressive airflow limitation that is not fully reversible with bronchodilators and corticosteroids, and an associated inflammatory response of the airways to noxious particles and gases (Barnes, 2003). Tobacco smoking is considered as the main etiological factor in western countries, but why only a minority of smokers (about 20 %) develop COPD is not known (Barnes, 2003; Barnes, Shapiro, & Pauwels, 2003; M. Saetta, 2006).

Thus, in COPD, there may be both airway inflammation and damage and destruction of lung parenchyma (Barnes et al., 2003). COPD is pathologically and physiologically complex with a number of overlapping and interacting components. The airway component includes chronic obstructive bronchitis characterised pathologically by inflammation of central airways and obstructive bronchiolitis, which is an inflammatory state involving peripheral small airways (<2mm in diameter), characterized by obliterated lumen with accumulation of inflammatory mucous exudates (Barnes, 2003; Szilasi, Dolinay, Nemes, & Strausz, 2006). Chronic bronchitis is a clinical entity of “chronic productive cough for 3 months in at least 2 consecutive years when there is no other reason for symptoms”(Mannino, 2003, 2007; Viegi et al., 2007). This reflects mucous hypersecretion and is not necessarily associated with airflow limitation though it frequently is (Barnes, 2003). The parenchymal component involves the process of emphysema and is a destructive part of the disease defined as “enlargement of the distal airspaces, beyond the terminal bronchioles, caused by destruction of the alveolar walls” (“The definition of emphysema. Report of a National Heart, Lung, and Blood Institute, Division of Lung Diseases workshop; MacNee, 2005).

One of the features of chronic airway diseases, including the airway component in COPD, is airway remodelling (Nakano et al., 2002), although this aspect of COPD has not been studied to the same extent as asthma. However, it is suggested that in COPD, remodelling may occur as a response to smoking-induced damage to the airways (Churg, Tai, Coulthard, Wang, & Wright, 2006; J. C. Hogg, Macklem, & Thurlbeck, 1968; Saetta et al., 2000), but the mechanism and structural changes are poorly described. A new explanation for remodelling in chronic airway disease is transition of airway epithelial cells to a mesenchymal phenotype with myofibroblast characteristics, through a process termed epithelial mesenchymal transition (EMT) (Peter K. Jeffery, 2004; Brigham C. Willis, duBois, & Borok, 2006). EMT was recently demonstrated in post transplant bronchiolitis obliterans syndrome (BOS) (Ward et al., 2005). BOS is characterised by neutrophilic airway inflammation, aberrant epithelial repair, airway remodelling and fixed airflow obstruction with some similarities to COPD, but develops over a much shorter time scale (Borthwick et al., 2009; Ward et al., 2005). It has been suggested that EMT could occur in COPD (Camara & Jarai, 2010), but there have been no studies undertaken specifically examining markers of EMT in human airway biopsy tissues from subjects with COPD.

In this chapter I am going to review a preliminary study on which much of the rest of my work depended. *Very early on, I identified that the central airway reticular basement membrane (Rbm) in current smokers with COPD was highly fragmented* (Figure 1.1), with cracks or elongated spaces (termed “clefts”) in the Rbm with cells within them, this was very striking and quite different to what we see in bronchial biopsies from asthmatic patients where Rbm is very homogenously thick and hyaline in appearance (Sagani et al., 2006), and further in addition to that literature strongly supported the fact that this can be a hallmark of EMT (Acloque, Adams, Fishwick, Bronner-Fraser, & Nieto, 2009; R. Kalluri, 2009; Raghu Kalluri & Neilson, 2003; R. Kalluri & Weinberg, 2009; Zeisberg & Neilson, 2009). These data are a fundamental platform for this thesis and explain that how this initial exciting observation led me to my hypothesis and research goals of the study.

This finding of Rbm fragmentation and strong relevant supporting information from the literature suggesting that such fragmentation is a hallmark of EMT encouraged

me to test the hypothesis that the airway epithelium is activated in current smokers with COPD and that this may promote EMT. After this original observation, my next phase was to design a pilot study in which I examined airway biopsies for expression of markers published on being associated with EMT including matrix metalloproteinase-9 (MMP-9), fibroblast specific protein 1, S100A4 and epidermal growth factor receptor (EGFR). MMP-9 can potentially digest type IV collagen (Ward et al., 2005) in the Rbm to potentially assist migration of cells from the epithelium through into the subepithelial lamina propria (Raghu Kalluri & Neilson, 2003; J. M. Lee, Dedhar, Kalluri, & Thompson, 2006; Ward et al., 2005). S100A4 is a very early indicator of fibroblast differentiation towards a mesenchymal phenotype (Ward et al., 2005) and has been considered as a very reliable marker to characterise mesenchymal phenotype (R. Kalluri & Weinberg, 2009). EGFR expression (marker of epithelial activation) can be involved in mediating phenotypic transition of epithelial cells into myofibroblasts and stimulating their migration (de Boer et al., 2006; Raghu Kalluri & Neilson, 2003). In this pilot study, I focussed on current smokers with COPD, essentially as a “test of concept”, I hypothesised that EMT would be most active in these individuals, and if nothing would be found, then there would be little point in carrying on to a large and more definitive study.

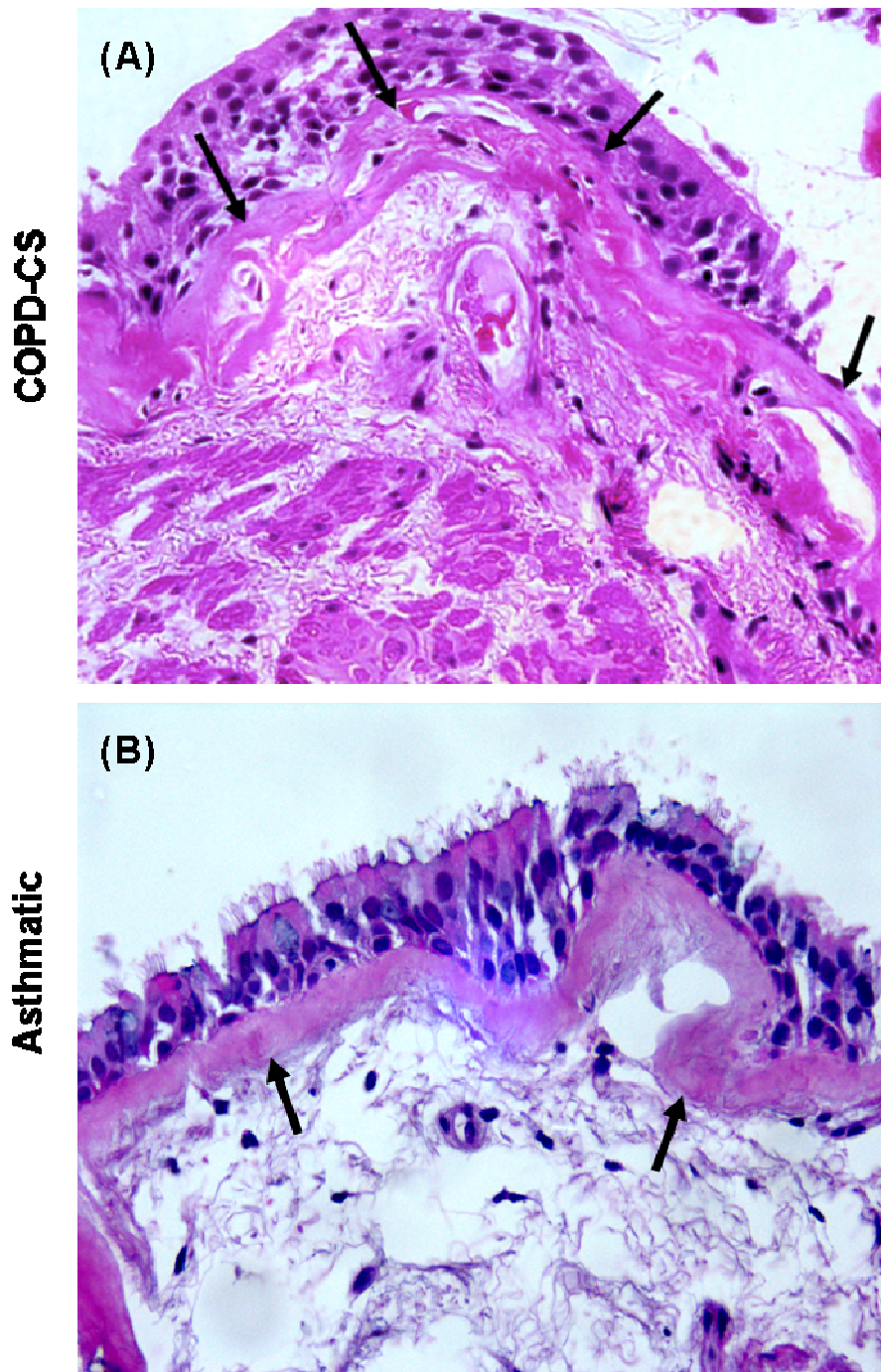


Figure 1.1: Bronchial biopsy specimen from (A) COPD current smoker, black arrows showing Rbm fragmentation with many “clefts” evident with cells in it, (B) non-smoker asthmatic showing thick homogenous Rbm with hyaline appearance. Stain: haematoxylin and eosin (H & E). Original magnification, x630

1.2 Materials and methods

Most of the details of my materials and methods are given in chapter 3, but the following is a brief summary of how I carried out the preliminary pilot study.

1.2.1 Subjects and study design

The study was a cross-sectional analysis of airway remodelling in COPD and normal subjects, using biopsies obtained at fiberoptic bronchoscopy. Study was approved by the Alfred and Royal Hobart Hospital Ethic Committees. Subjects were recruited by advertisement. 10 COPD current smokers and 12 normal non-smokers were recruited. All subjects signed informed consent. Subjects with a history of other respiratory disorders were excluded.

1.2.2 Details of biopsies and immunostaining

Details regarding processing of biopsies and immunostaining are given in chapter 3 and remained essentially unchanged throughout my thesis work.

1.2.3 Biopsy analysis

Image analysis was performed with a Leica DM 2500 microscope (Leica Microsystems, Germany), Spot insight 12 digital camera and Image Pro V5.1 (Media Cybernetics, USA) software, cellular plus other changes in the Rbm (Figure 1.1) were described in terms of the total length of basement membrane assessed. EGFR was measured as percentage of epithelium stained for EGFR. All slides were counted by an observer blinded to subject and diagnosis.

1.2.4 Statistical analysis

After counting of all specimens had been completed the data was analysed by group and only then unblinded. As data were not normally distributed, results are presented as medians and ranges. Comparisons between COPD and normal controls were undertaken using the Mann Whitney U test. Associations between variables were assessed using Spearman's rank test. Statistical analyses were performed using SPSS 15.0 for Windows, 2003, with a two-tailed p -value ≤ 0.05 being considered statistically significant. Details are given in Chapter 3.

1.3 Results of PILOT study

Table1: Demographic and lung function data for subjects.

	Non-smoking controls n = 12	COPD current smokers n = 10	Significance (<i>p Value</i>)
Male/female	5/7	7/3	
Age (years)	47(20-68)	61(53-69)	p = 0.02
Smoking (pack years)	0	46(32-79)	
FEV1% predicted	114(86-140)	74(66-96)	p < 0.001

Data are expressed as medians and range(s)

1.3.1 Fragmentation and presence of “clefts” within the Rbm

A very remarkable finding was that the Rbm in COPD current smokers was indeed extensively fragmented. This included the presence of the linear elongated spaces or cracks already mentioned that we called “clefts” in the Rbm which we used these as the index to quantitate the degree of fragmentation. However, there were other changes including fragments hanging off the Rbm or similar pieces separated from it (Figure 1.1). Fragmentation was rarely present in normal controls. The length of the individual “clefts” was measured, summed and results expressed as percentage total clefts length/total Rbm length to give a measure of the degree of disruption. This was significantly higher in the COPD subjects compared to normal controls (14 % (0-39) *versus* 0 % (0-9) $p < 0.001$) (Table 2).

1.3.2 MMP 9 positive cells in the Rbm

There were significantly more cells that stained positive in Rbm for MMP-9 in COPD subjects compared to controls, located predominantly within the “clefts” or adjacent and immediately below the Rbm (0.8 per mm (0-4.3) *versus* 0 per mm (0-0.9), $p = 0.003$) (Figure 1.2 & Table 2). There was minor MMP-9 staining of cells within the airway epithelium, but no significant difference between the COPD and normal subjects ($p = 0.7$).

1.3.3 S100A4 positive cells in the Rbm

Cells staining positively for S100A4 were found within the Rbm in COPD in the clefts, but not exclusively so, they were also found throughout the Rbm. The number of S100A4 positive cells within the Rbm was significantly higher in COPD subjects than in normal controls (44.1 per mm (28.1-92.6) *versus* 5.9 per mm (1.3-14.2) $p < 0.001$) (Figure 1.2 & Table 2). It was evident that there were also cells staining for S100A4 within the basal layer of the airway epithelium just superficial to the Rbm in subjects with COPD, but this was much less apparent in normal controls (26 per mm (7-37) *versus* 5 per mm (1-15) $p < 0.001$) (Figure 1.2 & Table 2).

1.3.4 EGFR expression in the airway epithelium

A greater proportion of the airway epithelium area stained significantly positive for EGFR in COPD subjects compared to normal controls (45 % (14 % -76 %) *versus* 6 % (0.6 %-17 %), $p < 0.001$) (Figure 1.2 & Table 2).

Table 2 Summary of immunohistochemical data for different markers

Significance	COPD		Non-smoking
	Current smokers n = 10	Controls n = 12	(p Value)
Clefts *	14(0-0.39)	0 (0-0.09)	$p < 0.001$
MMP-9 +ve cells **	0.8(0-4.3)	0 (0-0.9)	$p = 0.003$
Rbm S100A4 +ve cells***	44.1(28.1-92.6)	5.9 (1.3-14.2)	$p < 0.001$
Basal S100A4 +ve cells†	26(7-37)	5(1-15)	$p < 0.001$
EGFR % stain area	45.2 (14.4-76.1)	6.2 (0.6-17.7)	$p < 0.001$

Data expressed as median and (range) (26 (7-37) *versus* 5 (1-15) $p < 0.001$) per mm

* Values for clefts represent ratio of length of clefts to total length of BM

** Values for MMP-9 positive cells represent as number of cells counted in clefts per mm of BM

*** Values for S100A4 positive cells represent number of cells counted in Rbm per mm of Rbm

† Values for basal S100A4 positive cells represent number of cells counted in basal epithelium per mm of Rbm

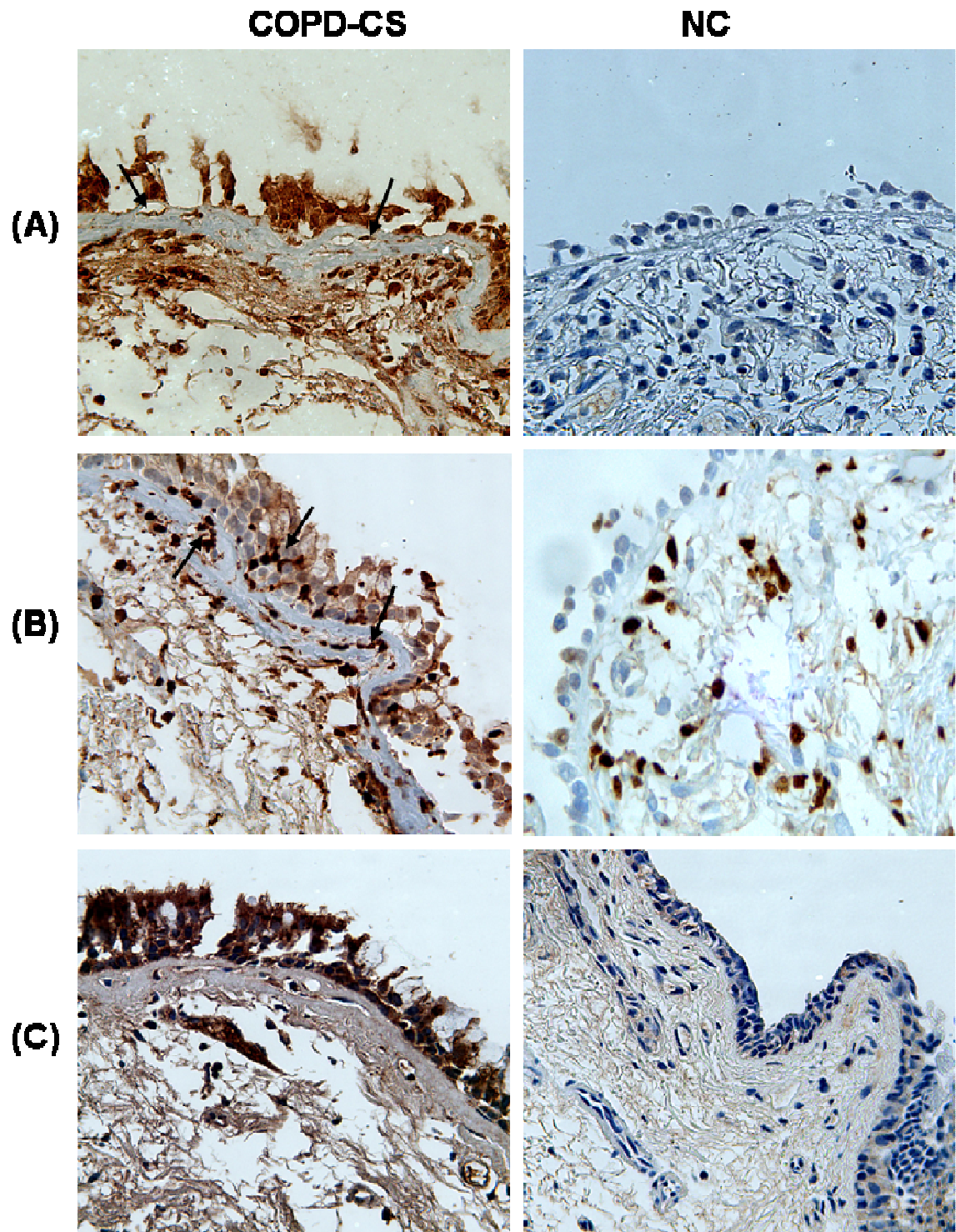


Figure 1.2: Immunohistochemical staining of bronchial biopsy sections from COPD-CS, compared to NC: (A) MMP-9, black arrows showing clefts containing cells positive for MMP-9, (B) S100A4, black arrows showing clefts, cells positive for S100A4 in the Rbm and basal cell S100A4 expression (C) intense brown staining for EGFR in the epithelium. Original magnification, x630

1.4 Preliminary discussion

This is the first description of marked Rbm pathological changes with extensive fragmentation in current smokers with established COPD. This included the presence of linear “clefts” or longitudinal spaces or cracks, which we decided pragmatically to use for quantifying the degree of fragmentation, but there were also commonly fragments of Rbm “hanging off” and similar pieces seemingly separated from it, albeit in the largely two-dimensional sections. “Clefts” within the disrupted Rbm contained cells expressing the proteolytic enzyme MMP-9 and the fibroblast marker S100A4. More superficial to the Rbm, the airway epithelium stained strongly for cells expressing the receptor for epidermal growth factor (EGFR). There was also marked staining with S100A4 in cells within the basal layers of the airway epithelium, situated in close proximity to the Rbm, in COPD subjects.

Rbm fragmentation has been suggested as the “hallmark” of EMT in the literature. Indeed, especially when associated with S100A4 positive cells such fragmentation is considered as one of the major *in vivo* criteria for identifying tissue undergoing EMT. From a general prospective EMT basically involves epithelial cells disaggregating, changing shape, becoming motile and digesting their way through basement membrane collagen into the subepithelial layer, while at the same time they also develop a fibroblast phenotype (Raghu Kalluri & Neilson, 2003). The process involves loss of epithelial markers such as tight junctions, apical-basal polarity and specific markers such as cytokeratins and E-cadherins, and this is replaced with gain of migratory potential, irregular cell shape and expression of mesenchymal markers cell markers such as matrix metalloproteinases, vimentin and S100A4 (Raghu Kalluri & Neilson, 2003; J. M. Lee et al., 2006; Ward et al., 2005; Brigham C. Willis et al., 2006). EMT has only recently been recognised in the human lung in idiopathic pulmonary fibrosis (Brigham C. Willis et al., 2006) and in the airway in BOS (Ward et al., 2005), but it is well described in lung embryogenesis (J. M. Lee et al., 2006), metastatic malignant disease (Bjornland et al., 1999) and as part of the repair process in renal disease following tissue injury (Yanez-Mo et al., 2003).

EMT has been described in metastatic malignant disease especially when associated with S100A4 expression. Nuclear expression of S100A4 strongly correlates with

both active EMT and metastatic disease in the oncology literature (Flatmark et al., 2003; Ward et al., 2005). In particular, in the context of lung diseases, increased expression of both S100A4 and MMP-9 are observed in human non-small cell lung cancer and are significantly correlated with clinical and biological behaviour of this cancer cells (Chen, Wang, Zhang, Chen, & Sun, 2008). Further, EGFR is also over-expressed in many types of cancers, including non-small cell lung cancer. It has now been shown that sensitivity to EGFR inhibition using the “biologic” monoclonal antibody erlotinib is directly linked to the degree of EMT in this lung cancer. It is clear from these data that EGFR over-expression is an important aspect of the lung cell cancer phenotype (Dasari, Gallup, Lemjabbar, Maltseva, & McNamara, 2006) and that active EMT is potentially important in the pathogenesis of lung cancer and its prognosis.

The link between EMT and lung cancer needs to be placed in the context of the well-described association between COPD and lung cancer. Although, a proportion of this may be related to a common exposure to tobacco smoke, it has become clear that for any level of tobacco exposure, patients with COPD have a substantially greater risk for lung cancer than smokers without COPD (Parimon et al., 2007). Thus, my finding of active EMT in smokers, which is especially active in COPD current smokers, may well advance understanding of why lung cancer is so common and aggressive in smokers. In this context it is an aggressive and invasive tumour which is fatal in over 80% of cases.

One of the features of chronic inflammatory airway diseases, including COPD, is airway remodelling. There are several known features of airway remodelling in COPD including; metaplasia of the epithelium, loss of epithelial cilia, an increase in goblet cell size and number (Saetta et al., 2000) and hypertrophy of submucosal mucus glands. These were first described 40-50 years ago by Professor Lynne Reid in London. In contrast to asthma, there is much less information in COPD on changes to the basement membrane and extracellular matrix. There aren't any studies reporting potential presence of EMT in COPD; this is the first suggestion that EMT may be active in COPD. However, there is evidence of EMT in patients after lung transplantation and it is suggested that there may be a potential link between EMT and airway remodelling leading to development of BOS (Borthwick et al.,

2009). So as a simile to the EMT suggested in BOS (Borthwick et al., 2009; Ward et al., 2005) it is possible that in COPD, the epithelium gets damaged and/or activated by cigarette smoke constituents, which may lead to airway remodelling, a part of which is related to active EMT.

Our finding of Rbm fragmentation in COPD current smokers is novel. However, in retrospect we have now looked for and noted Rbm fragmentation in micrographs published by other investigators, (de Boer et al., 2006) but this has not previously been commented on to our knowledge. Within the “clefts” in the Rbm, we observed cells expressing MMP-9 and S100A4 and we suggest that these cells have originated from the surface airway epithelial layer. It is also possible that the cells we identified in clefts may have migrated from the lamina propria, but against this explanation was the presence of S100A4 staining cells in the basal layer of the epithelium in COPD, and the accompanying marked increase in expression of EGFR in the epithelium. However, in chapter 5, I went on to show that these cells were not infiltrating inflammatory cells.

In summary, this preliminary “pilot”, test of concept, study demonstrated that the Rbm in subjects with COPD who are current smokers is highly fragmented and that this appears to be associated with cells expressing the proteolytic enzyme MMP-9. Epithelial expression of EGFR suggested that epithelial cells may be priming themselves for migration, and increased expression of the fibroblast marker S100A4 suggests phenotypic transition. These preliminary data suggested that EMT could be active in COPD pathology, and needed following up in a more definitive set of studies, asking the following questions:

1) At this stage it was very hard to say whether these observations represented underlying pathology of COPD *per se* or whether they simply reflected the general effects of cigarette smoking. To address this issue, we needed to stain biopsy sections from normal lung function smokers without physiological evidence of COPD for the chosen markers of EMT to help us define whether this is a smoking effect or it is related to COPD, or indeed both i.e. additive.

2) *Another key question* which needed to be answered was whether these changes are plastic over time, especially with quitting smoking? In the first instance I chose to have a COPD ex-smoker group to help us to understand whether smoking cessation has any potential for decreasing EMT or not.

3) *Is Rbm fragmentation normalised with ICS therapy?*

4) *I addressed* the need to confirm the epithelial origin of the cells we identified in the Rbm (positive for S100A4 and MMP-9). To do this I needed to double-stain biopsies for an epithelial marker such as cytokeratins and with a mesenchymal marker such as S100A4 and/or vimentin. This would be additional evidence to my finding of basal epithelium cells positive for S100A4.

5) *The last question* which needed to address, was the possibility that these cells I identified in the Rbm could be infiltrating inflammatory or structural cells, such as neutrophils, macrophages, fibroblasts, dendritic cells, CD4+ or CD8+ T cells or B-cells, for which I needed to stain biopsy sections for their specific markers.

So in summary, after the pilot study, I needed a comprehensive cross-sectional analysis of these new features of airway pathology and putative markers of EMT in currently smoking physiologically normal subjects, as well as in reformed smokers, especially those with and without COPD to confirm and extend these preliminary but novel and exciting findings into a longitudinal analysis to investigate reversibility of Rbm changes with ICS therapy.

As a part of my thesis, but not directly related to my preliminary study which I have outlined in this chapter, I also decided early on to study the status of histone deacetylase-2 (HDAC2) in COPD. It is not well understood why COPD responds relatively poorly to some therapies compared to asthma, especially the relative resistance to the anti-inflammatory effects of corticosteroids. The molecular mechanism behind “corticosteroids resistance” in COPD, although perhaps exaggerated by Peter Barnes and others, is now being revealed and anti-inflammatory effects of steroids are now better understood (Barnes, 2006b; Barnes & Stockley, 2005). These workers have suggested that corticosteroid resistance in

COPD is due to a decrease in the key nuclear enzyme HDAC-2 (Barnes, 2006c; Ito et al., 2001), but the reason behind HDAC-2 reduction in COPD is not clear, although it is suggested that increased oxidative and nitrative stress in the airways due to cigarette smoking might lead to inactivation or destruction of the enzyme (Barnes, Adcock, & Ito, 2005). In addition, the expression of anti-inflammatory HDAC2 may be reduced in COPD due to increased oxidative stress secondary to airway inflammation and this may induce (relative) resistance to inhaled corticosteroid therapy, but there is little confirmatory data on this, especially outside of the Peter Barnes group in London, which is highly committed to this theory. Furthermore, I hypothesized that reduced HDAC2 levels could be normalised by aggressive ICS therapy and smoking cessation in patients with COPD.

To test these questions and address these issues, I designed a comprehensive cross-sectional and longitudinal study (for longitudinal analysis I used bronchial biopsies from the study previously designed (D. W. Reid et al., 2008) by our group):

Firstly, I looked at the extent of HDAC2 suppression by immunostaining airway biopsy sections from COPD current smokers and also from normal lung function smokers to understand whether HDAC changes are solely related to COPD or smoking or both.

Secondly I wished to investigate the potential of smoking cessation to raise HDAC2 levels, so I included COPD ex-smokers.

Finally, to see if HDAC2 levels are normalised by aggressive ICS therapy, I used material from the study in which COPD subjects entered a double blind randomised controlled trial comparing fluticasone propionate (0.5 mg/twice daily) with placebo for 6 months (D. W. Reid et al., 2008); I assessed the bronchial biopsies which had been obtained before and after treatment for effects of ICS on HDAC2. This is the same study in which I investigated ICS effects on Rbm fragmentation.

In summary, my thesis involves a comprehensive cross-sectional analysis into the potential for active EMT in COPD, whether it is specific to COPD and/or smoking or both, and a longitudinal analysis to see if ICS therapy has any potential for reducing Rbm fragmentation. The second part of my thesis involves a cross-sectional and longitudinal analysis questioning the status of HDAC2 in COPD and asking whether smoking cessation and/or ICS therapy have any potential to raise HDAC2 levels in COPD.

Chapter 2

General Introduction

2.1 Definition of COPD

“COPD is a disease state characterized by airflow limitation that is not fully reversible, usually progressive and associated with an abnormal inflammatory response of the lung airways in response to noxious particles and gases” (Barnes, 2003).

The term “chronic obstructive pulmonary disease” (COPD) now widely used was first used in the literature in 1964 (Mitchell & Filley, 1964). Later on in the 1970s and 1980s, sub-diagnosis such as emphysema, chronic bronchitis, chronic obstructive bronchitis and chronic bronchitis with emphysema were used and shortly recommendations and international guidelines became available on how to use these terms to define the disease, today called COPD (Larsson, 2007; Mitchell & Filley, 1964). Tobacco smoking is considered as the main etiological factor in this condition, at least in western countries. Why only a minority of smokers develop COPD is not known.

A number of different specific definitions exist for COPD in the literature but they are of course more or less similar. *The American Thoracic Society (ATS)* define COPD as “a disease state characterized by the presence of airflow limitation due to chronic bronchitis or emphysema; the airflow obstruction is generally progressive, may be accompanied by airway hyper-reactivity, and may be partially reversible” (“Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. American Thoracic Society,” 1995). This, therefore, allows some overlap with asthma.

The European Respiratory Society (ERS) on the other hand defines COPD as “reduced maximum expiratory flow and slow forced emptying of the lungs, which is slowly progressive and mostly irreversible to present medical treatment” (Siafakas et al., 1995).

The Global Initiative for Chronic Obstructive Pulmonary Lung Disease (GOLD) report classified COPD as “a disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases” (GOLD, 2007; Gomez & Rodriguez-Roisin, 2002; Pauwels, Buist, Ma, Jenkins, & Hurd, 2001). GOLD definition is the most widely recognised definition of COPD worldwide and is somewhat different in that it introduces an underlying pathological process. The GOLD criteria also further categorized COPD into four different stages on the basis of the physiological and clinical severity of disease as given below:

“*GOLD Stage I* is considered as mild COPD, characterized by mild airflow limitation ($FEV_1/FVC < 0.70$; $FEV_1 \geq 80\%$ predicted). Symptoms of chronic cough and sputum production may be present, but not always. At this stage, the individual is usually unaware that his or her lung function is abnormal” (taken from www.goldcopd.com).

“*GOLD Stage II* is considered as moderate COPD, characterized by worsening of airflow limitation ($FEV_1/FVC < 0.70$; $50\% \leq FEV_1 < 80\%$ predicted), with shortness of breath typically developing on exertion and cough and sputum production sometimes also present. This is the stage at which patients typically seek medical attention because of chronic respiratory symptoms or an exacerbation of their disease” (taken from www.goldcopd.com).

“*GOLD Stage III* is severe COPD, characterized by further worsening of airflow limitation ($FEV_1/FVC < 0.70$; $30\% \leq FEV_1 < 50\%$ predicted), greater shortness of breath, reduced exercise capacity, fatigue, and repeated exacerbations that almost always have an impact on patient’s quality of life” (taken from www.goldcopd.com).

“*GOLD Stage IV* is very severe COPD, characterized by severe airflow limitation ($FEV_1/FVC < 0.70$; $FEV_1 < 30\%$ predicted or $FEV_1 < 50\%$ predicted plus the presence of chronic respiratory failure). Thus, patients may have *Stage IV: Very severe COPD* even if the FEV_1 is $> 30\%$ predicted where secondary complications are present. At this stage, quality of life is very appreciably impaired and exacerbations may be life threatening” (taken from www.goldcopd.com). Prognosis at this stage is also poor.

As included in ATS definition, COPD includes two distinct entities, chronic obstructive bronchitis characterized pathologically by airway inflammation and airway damage, and emphysema which consists of enlargement of peripheral airspaces and destruction of lung parenchyma, leading to loss of lung elasticity which then causes dynamic closure of small airways in expiration. “Chronic bronchitis” is a clinical entity defined symptomatic features of “chronic productive cough for 3 months in each of 2 consecutive years provided other causes of cough have been ruled out” (Mannino, 2003, 2007; Viegi et al., 2007). This reflects mucous hypersecretion and is not necessarily associated with airflow limitation though it frequently is (Barnes, 2003). There is no exact clinical definition of “emphysema” apart from the anatomical definition, but “clinically patient’s experiences progressive dyspnea and variable cough” (Mannino, 2003, 2007). However, it is reflected physiologically in decline in gas-absorption function of the lungs as reflected in measurement of the CO-transfer factor (diffusion capacity).

2.1.1 Social and economic burden

COPD is one of the leading causes of disability and death worldwide and is the fourth most common cause of death in the United States, Australia and Europe. In Australia nearly 12 % of the entire adult population is affected by the disease and due to inadequate diagnosis in its early stages related to its slowly progressive and insidious nature its prevalence is probably much higher (D. W. Reid et al., 2003). It is estimated that over one million individuals in the UK, 6% of men and 4% of women, suffer from COPD with around about 30,000 deaths annually and is anticipated that it will rank fifth by 2020 in burden of disease worldwide, according

to a study published by the World Bank/World Health Organization (Barnes, 1998b; Rabe et al., 2007; Thorley & Tetley, 2007).

COPD has very major social and economic consequences (Johnson, Campbell, Bowers, & Nichol, 2007). Thone and colleagues (Thone, Schurmann, Kuhl, & Rief) in a very interesting study reported spouses' quality of life during the course of the disease. They showed that spouses also had poor quality of life and with increased psychological suffering. It has also been observed that patients with COPD feel disgrace and distancing from people around them and also from their physicians (Johnson et al., 2007). A study reported by Earnest and colleagues described patients feeling personal shame and embarrassment when they are carrying oxygen cylinders with them in public (Earnest, 2002).

The European Lung White Book in 2001 evaluated exhaustively the annual cost associated with COPD in Europe; they found that COPD costs €38.7 billion per annum (A\$ 54.4 billion) including €4.7 billion (A\$ 6.6 billion) for ambulatory care, €2.7 billion for drugs (A\$ 3.7 billion), €2.9 billion (A\$ 4.08 billion) for inpatient care and €28.4 (A\$ 39.9 billion) for lost work days very year. The estimated cost for individual European countries annually ranged from €109-€541 million (A\$153-A\$761 million), and individual patient costs were estimated around from €151-€3,912 annually (A\$ 212-A\$5504) (Chapman et al., 2006; Loddenkemper R, 2003). The economic burden associated with COPD is probably underestimated as well, because the economic value of the care provided by family members of patients with COPD is not usually reported. A patient with very severe COPD needs long term home care which has serious negative effects not only on their own professional life but also on the life of engaged relatives and carers (Viegi et al., 2007).

2.1.2 Risk factors associated with COPD

Direct exposure to tobacco smoke is considered as the main risk factor associated with progression of COPD in western countries. The cigarette smoke which is inhaled while puffing is approximately 45% of the total while the remaining 55% is released into the surrounding environment (Yoshida & Tuder, 2007). Environmental tobacco exposure or "passive smoking" (Pinkerton & Joad, 2006; Sethi & Rochester,

2000; Yoshida & Tudor, 2007) is a growing centre of attention, given that such lifetime exposure could be associated with COPD (Eisner et al., 2005). The increasing burden of indoor pollution due to biomass fuel smoke (Fullerton, Bruce, & Gordon, 2008), outdoor air pollution, viral infections and occupational exposure to noxious gases and dust may also cause COPD (Blanc et al., 2009; Driscoll et al., 2005). Biomass fuel refers to burned animal or plant material; charcoal, wood, and even cow dung and crop residues, which provide more than one-half of domestic energy in most of the developing world and nearly 95 % in low income countries (Fullerton et al., 2008). There is evidence in the literature now, and more data is accumulating, explaining the potential link between an increased risk of respiratory tract infections, inflammatory lung / airway diseases and increased levels of indoor air pollution especially due to increase in biomass fuel consumption in rural areas (Fullerton et al., 2008; Jaakkola & Jaakkola, 2006). Understanding the effects of burning biomass fuels on health is important for any physician practising in the developing world, but unfortunately not well represented in the literature and it is quite surprising how little published research there is to date on its health consequences (Jaakkola & Jaakkola, 2006). There are few studies reporting associations between biomass fuel combustion and physiological and structural pathological changes in the respiratory system and little hard data are available on relationships between biomass fuel consumption and COPD. However, a Chinese study reported that biomass fuels are the probable primary risk factor for COPD in rural South China; they found that the use of biomass fuel was higher in rural areas, with a strong association between COPD and exposure to biomass fuel used for cooking. They also reported higher concentrations of carbon monoxide, particulate matter, sulphur dioxide and nitrogen dioxide in the kitchen during biomass fuel combustion compared to LPG combustion (Liu et al., 2007).

In another study in Spain, Orozco and colleagues reported a strong association between wood or charcoal smoke exposure and COPD, suggesting that this is also a problem in less developed European countries (Orozco-Levi et al., 2006). In a systematic review and meta-analysis, Kurmi et al reported that even with enormous heterogeneity across different studies, exposure to biomass fuel is consistently associated with COPD and chronic bronchitis (Kurmi, Semple, Simkhada, Smith, & Ayres).

Despite the recognition of these environmental factors, the major cause of COPD worldwide/non-westernised countries is still a matter of debate (M. Roth, 2008). However, Salvi and Peter Barnes suggested in a recent review in the Lancet that about 3 billion people, which is nearly half of the total world population, are exposed to smoke from biomass fuel compared with 1.01 billion people who smoke tobacco, which suggests that exposure to biomass smoke, might be the biggest risk factor for COPD globally compared to tobacco smoke (Salvi & Barnes, 2009). However in westernised countries tobacco smoking is considered as the main etiological factor associated with COPD, but why only a minority of smokers develop COPD while others not, leads to a possibility that there may be a genetic susceptibility associated with COPD development interacting with environmental factors. Deficiency of protease inhibitor, alpha-1 antitrypsin is the only confirmed genetic risk factor associated with emphysema (Babusyte, Sitkauskienė, & Sakalauskas, 2006; Silverman, 2001), but this is a relative rare cause. Further discussion of the genetics of COPD is beyond the scope of this thesis although it is an area of much active research.

2.1.3 Tobacco smoke, components and airway epithelium

Cigarette smoke is a very diverse mixture of more than 4,700 different chemical compounds with a high concentration of free radicals and different oxidants and consists of a gas and a particulate phase or tar phase (MacNee, 2000). Free radicals are part of both gas and particulate phase in tobacco smoke. The gas phase of the cigarette smoke contains roughly 10^{15} radicals per puff mainly from alkyl and peroxy categories. Nitric oxide is another main component of cigarette smoke, present at concentration of 500 to 1000 ppm, which has the capacity to react with superoxide anion to form peroxynitrites and can also react with peroxy radicals to form alkyl peroxynitrites (MacNee, 2000; Pryor & Stone, 1993). Both peroxynitrite species are highly chemically reactive. The particulate phase, on the other hand, has more stable radicals like semiquinone radicals, hydrogen peroxide and hydroxyl radicals (Pryor & Stone, 1993), which add substantially to the oxidant burden.

The airway epithelium is one of the first tissue targets of cigarette smoke, which leads to the activation of a number of biological and cellular pathways characterising the development of COPD (Figure 2.1 and 2.2) (K. F. Chung & Adcock, 2008). Chronic exposure to reactive oxygen species (ROS) can cause lipid peroxidation in airway epithelium leading to disruption of cellular membranes and inactivation of membrane-bound receptors and enzymes (Rahman, 2003, 2005; Rahman, Marwick, & Kirkham, 2004). Cigarette smoke induces epithelial changes related to development of chronic bronchitis in large airways which may or may not be associated with COPD, depending on the degree and/or outcomes of epithelial inflammation (J. C. Hogg, 2006). It can lead to mucus hypersecretion which is a feature of both large and small airways in COPD. Mucus secreting pathways are associated with EGFR over-expression in the airway epithelium in response to cigarette smoke-generated reactive oxygen species (Figure 2.3). Over expression of EGFR is also considered as one of the earliest abnormalities found in smokers at high risk of developing lung cancer (K. F. Chung & Adcock, 2008; Mutch, 2002). In small airways, cigarette smoke induces goblet cell metaplasia (Saetta et al., 2000) and increased connective tissue in the airway wall (James C. Hogg et al., 2004).

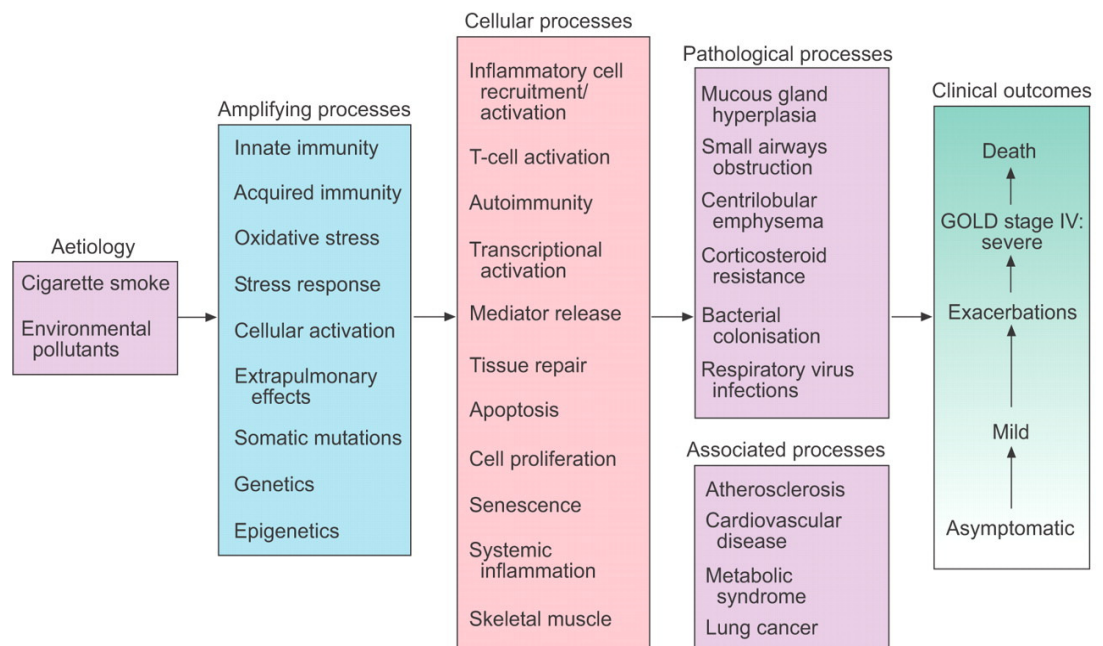


Figure 2.1: Showing link between aetiology of COPD and clinical outcomes (K. F. Chung & Adcock, 2008)

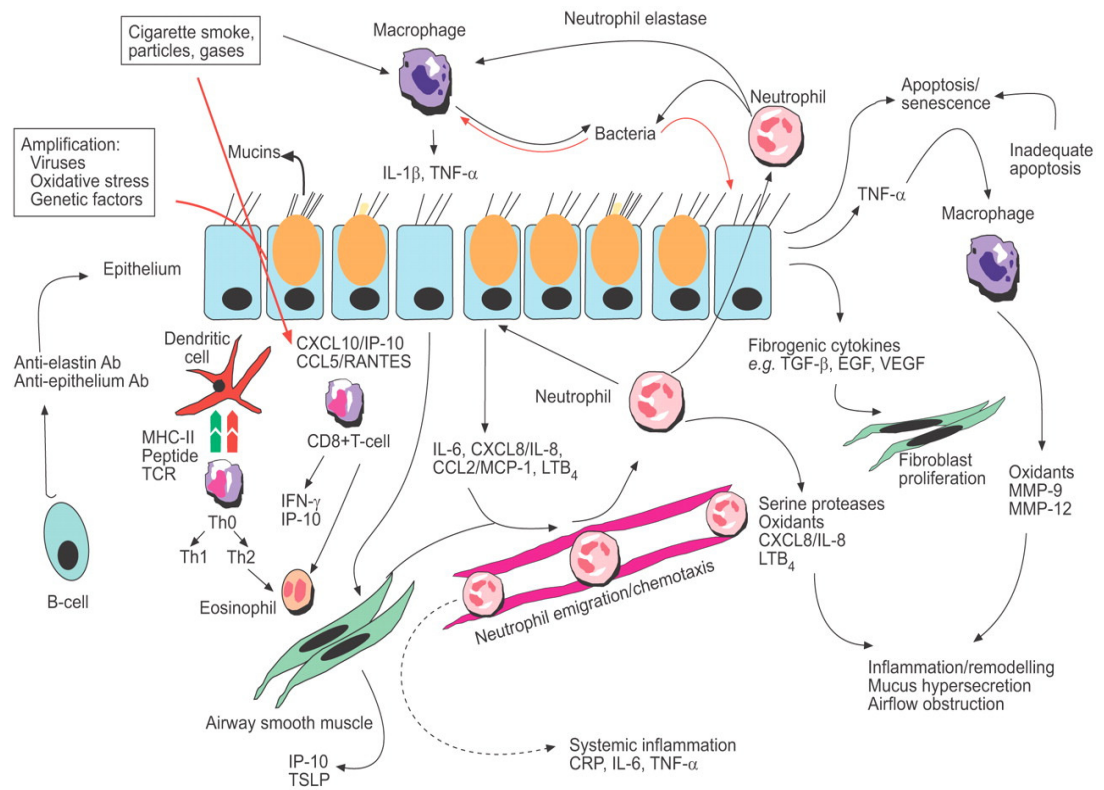


Figure 2.2: Outlining different inflammatory and cellular interactions linking chronic cigarette smoke exposure to chronic inflammation in COPD (K. F. Chung & Adcock, 2008).

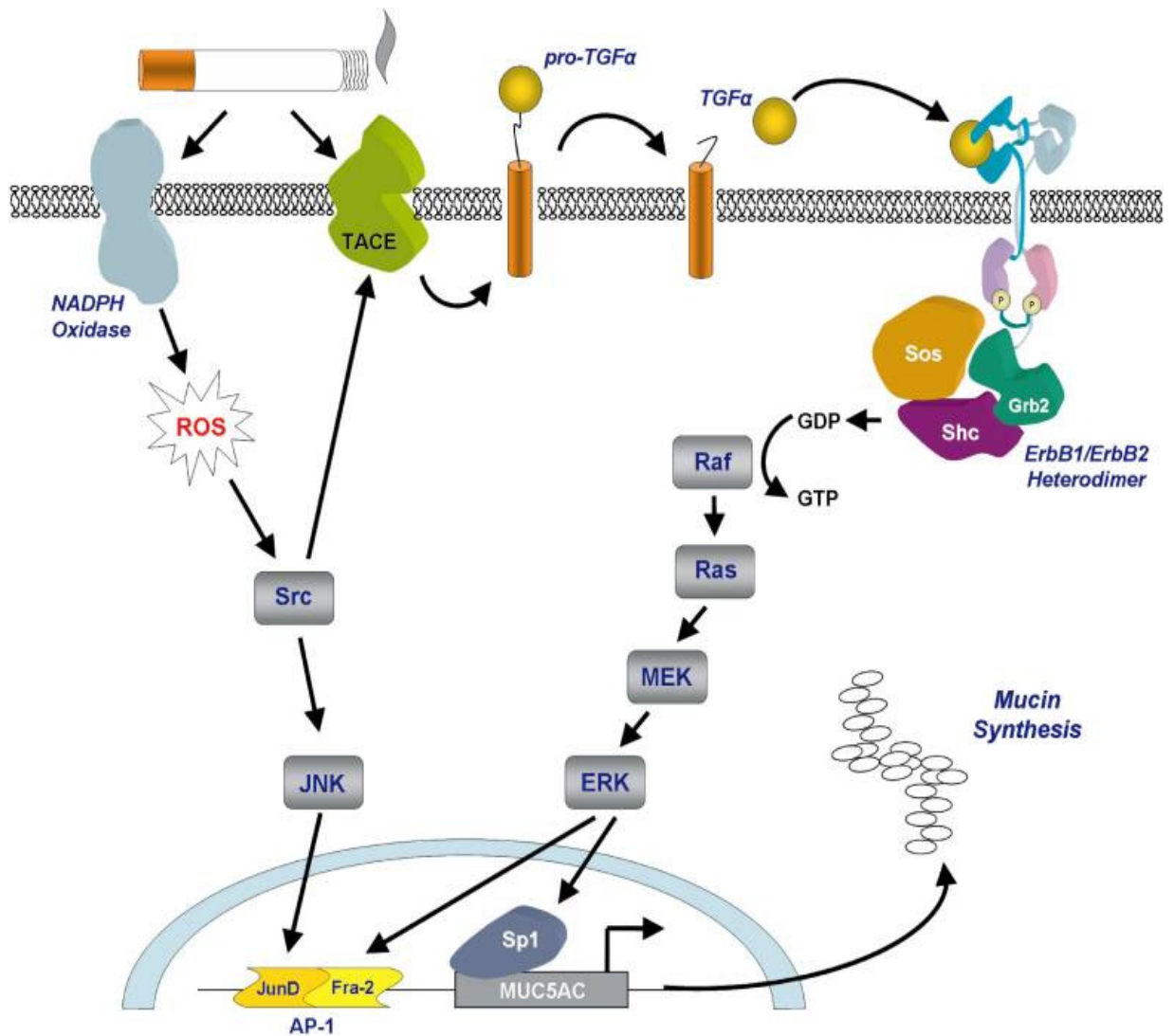


Figure 2.3: Mechanism of cigarette smoke induced mucin secretion in COPD (Thorley & Tetley, 2007).

Increased oxidative stress due to cigarette smoke inhalation also initiates the process of airway remodelling which is defined as alterations in structural components of the airways leading to (gross) thickening of the airway wall causing airway obstruction (P. K. Jeffery, 2001b). Increased oxidative stress also disturbs the protease-antiprotease balance of the lung, activates macrophages and neutrophils (both of which can secrete various proteolytic enzymes) and also CD8+ T cells which release perforin (cytolytic protein) and granzymes (sreine proteases). In addition oxidative

stress decreases VEGF signalling and alveolar repair and increased apoptosis of cells is also observed. All these pathways are associated with development of emphysema (Figure 2.4) with increased proteolytic load leading to elastic fibre damage (Kasahara et al., 2001; Morissette, Parent, & Milot, 2007; Sharafkhaneh, Hanania, & Kim, 2008).

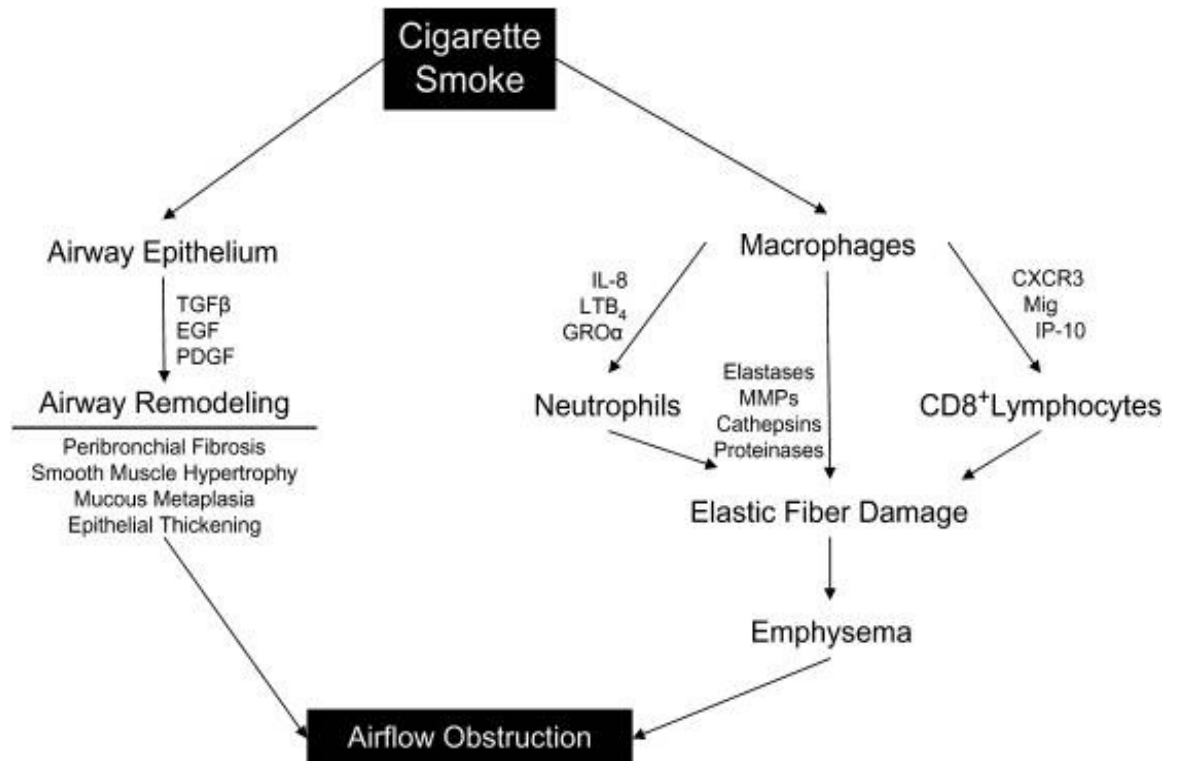


Figure 2.4: Mechanism of cigarette smoke induced airway remodelling and emphysema (Sharafkhaneh et al., 2008).

Numerous studies have reported changes in gene expression in epithelial cells in smokers and non-smokers (Carolan et al., 2006; Chari et al., 2007; N. R. Hackett et al., 2003; Harvey et al., 2007; K. M. Lee et al., 2007). Epithelial cells from small airways demonstrated an increase in the activation of pro-apoptotic gene p21, and in anti-oxidant genes including glutathione peroxidase, and ubiquitin carboxyl-terminal hydrolase (UCH) L1, which is a member of ubiquitin proteasome pathways, but in

contrast the expression of IL-4 receptor, CX3C chemokine ligand (CX3CL) 1 and the extracellular matrix protein spondin 2 were inhibited (Harvey et al., 2007).

Genes up-regulated during current smoking and then down-regulated with smoking cessation included those for trefoil factor 3, calcium-binding tyrosine-(Y)-phosphorylation-regulated protein, CXCL6, CX3CL1 and S100 calcium-binding protein A9 (S100A9). Partial reversibility with quitting was shown for genes transcribing for mucin (MUC) 5 subtypes A and C (also called as MUC5AC), but an irreversible gene was that for glycogen synthase kinase 3 β . The authors suggested that irreversible changes may account for the persistent lung cancer risk despite smoking cessation (Chari et al., 2007).

2.2 Pathology of COPD

The pathological changes in lungs associated with COPD essentially involve two anatomical compartments; the airway component of the disease, and that involving the parenchyma. They frequently coexist, but with some people having predominantly the airway component of the disease and some the parenchymal. The airway component includes inflammation of the central airways and small (peripheral) airways, and the parenchymal component of the disease involves the destructive process of emphysema.

2.3 Airway component

2.3.1 Central airways

Tobacco smoking is a critical factor in the pathological changes and inflammatory response associated with central airways in COPD. Inflammation may present as “chronic bronchitis”, a clinical-epidemiological entity defined by cough and sputum production which reflects mucous hypersecretion in response to tobacco smoke, noxious gases and dust which may not be associated with airflow limitation (Barnes, 2003; J. C. Hogg, 2004). Pathologically, in COPD when associated with airflow limitation, chronic bronchitis involves mucus gland hypertrophy and mucus hypersecretion, squamous metaplasia, loss of epithelial cilia, goblet cell hyperplasia

and hypertrophy, and infiltration of airway wall with inflammatory cells (Figure 2.5) (Caramori et al., 2004 32; Saetta et al., 2000 143).

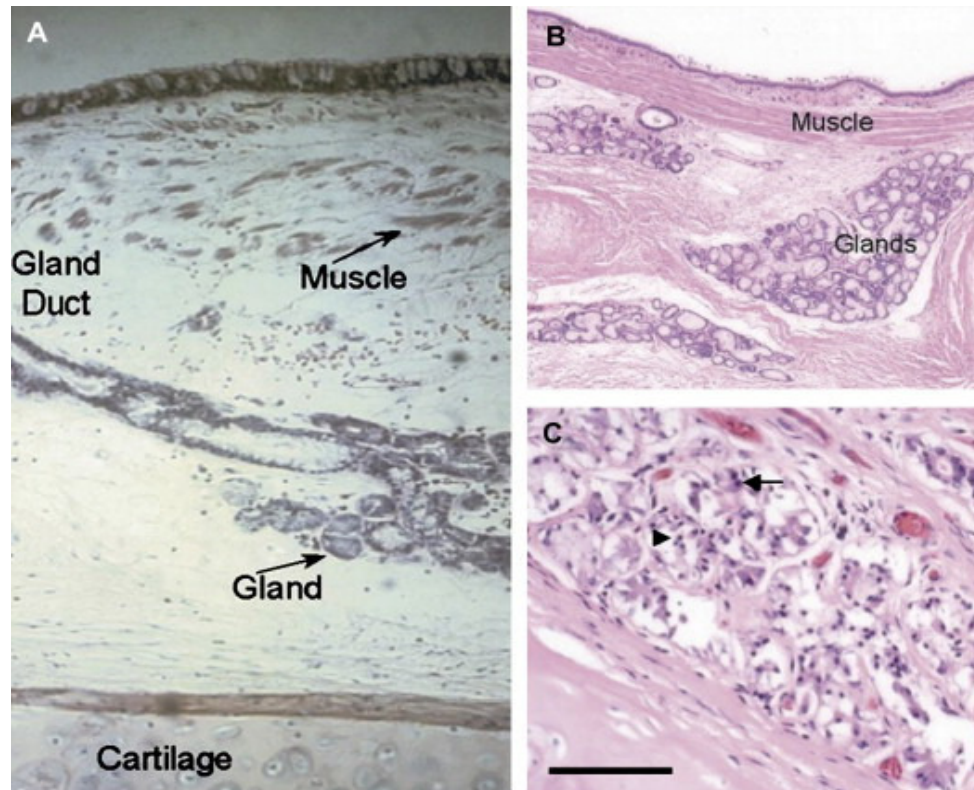


Figure 2.5: Pathologic changes of the central airways in COPD. (A) Central bronchus from the lung of a physiologically normal current smoker, which shows that only small amounts of bronchial smooth muscle are present and the glands are small, compared to a subject (B) with chronic bronchitis with thick bronchial smooth muscle and enlarged bronchial glands. (C) The enlarged bronchial glands at a higher magnification. It also indicates a chronic inflammation involving leukocytes (see arrows) and mononuclear cells, including plasma cells (see arrows) (Macnee, 2007).

Inflammation observed in airways/lungs from patients with COPD is multifaceted and not well understood. In chronic bronchitis inflammation is mainly associated with the epithelium of the larger airways and extends to mucus glands, and involves infiltration of neutrophils and macrophages with increased number of CD8⁺ T-lymphocytes in the bronchial glands (Mullen, Wright, Wiggs, Pare, & Hogg, 1985; Saetta et al., 1997).

Mucus gland hypertrophy was first described in 1954 by Reid (L. M. Reid, 1954), she observed that bronchial glands were enlarged in chronic bronchitis and used the ratio of mucosal gland to the thickness of bronchial wall for the diagnosis of chronic bronchitis, known as Reid's index (Caramori et al., 2004; L. Reid, 1960; L. M. Reid, 1954). Mucus hypersecretion associated with chronic bronchitis is still a matter of debate as to whether it is related to airway flow limitation or not (Maestrelli, Saetta, Mapp, & Fabbri, 2001).

It has been suggested that up-regulation/activation of the epidermal growth factor receptor (EGFR) on epithelial cells may be associated with abnormal epithelial cell growth and proliferation leading to goblet cell hyperplasia and hence to mucus hypersecretion in COPD (de Boer et al., 2006; Innes et al., 2006). Cigarette smoke is considered the main etiological factor associated with COPD and in humans chronic exposure to tobacco smoke may activate EGFR signalling pathway leading to mucus hypersecretion (Yoshida & Tudor, 2007).

2.3.2 Small airways (obstructive bronchiolitis)

COPD is widely accepted as a pan airway disease, but most attention has been given to the small airways (<2mm in diameter) where most of the obstruction occurs, characterized by an obliterated lumen with accumulation of inflammatory mucous exudates (Figure 2.6) (Szilasi et al., 2006).

Macklem and colleagues in 1967 introduced the idea of peripheral or small airway disease (J. C. Hogg, 2006; Macklem & Mead, 1967). Soon after, Hogg and colleagues suggested that small airways represent the “quiet zone” where disease can progress for a long time without being clinically diagnosed (James C. Hogg et al., 2004; J. C. Hogg et al., 1968; M. Saetta, 2006). The key pathological changes related

to progression of COPD in small airways involve inflammatory cell infiltration (James C. Hogg et al., 2004; Saetta et al., 1998), goblet cell metaplasia (Saetta et al., 2000) and increased connective tissue in the airway wall (James C. Hogg et al., 2004).

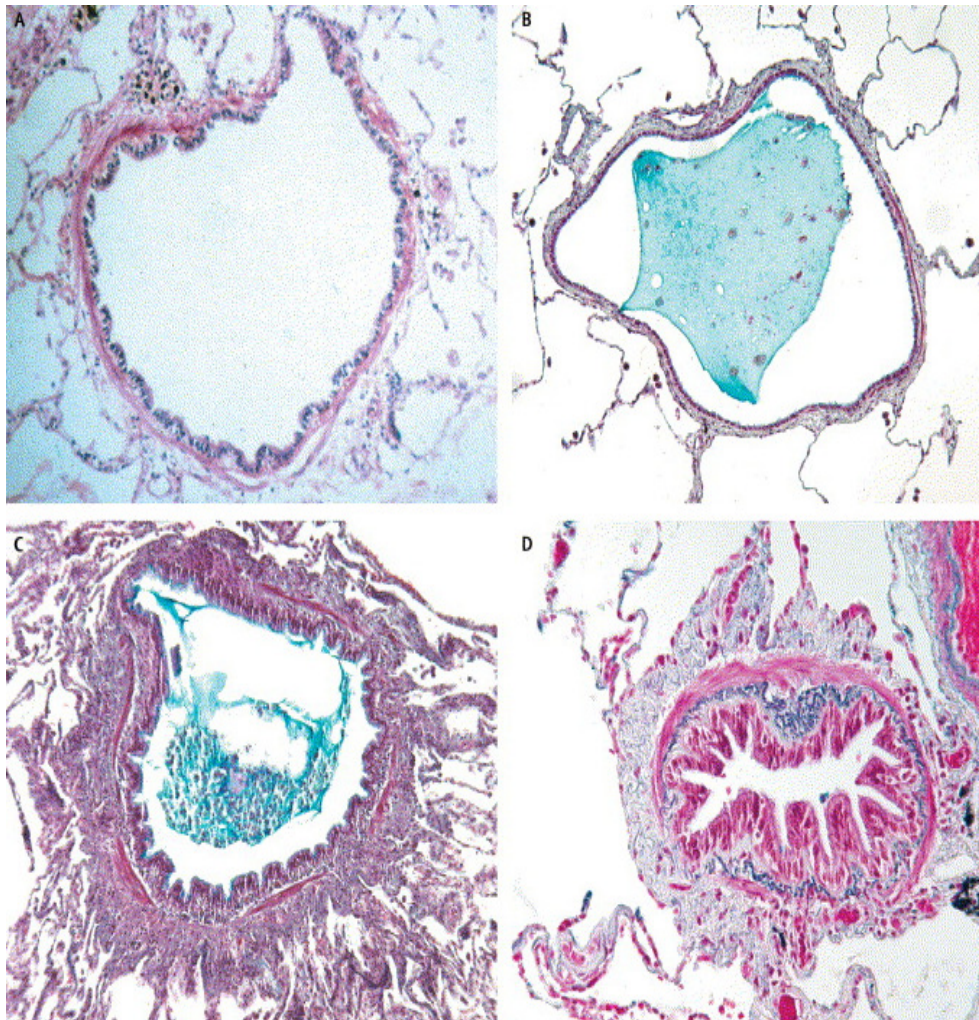


Figure 2.6: Small airway obstruction

(A) Normal small airway. (B) Small airway containing plug of mucus (C) Acutely inflamed airway with thickened wall in which the lumen is partly filled with an inflammatory exudates of mucus and cells (D) Airway surrounded by connective tissue and B cell follicles (J. C. Hogg, 2004).

Severe obstruction is associated with presence of B cells, CD4 and CD8 lymphocytes organized into follicles (James C. Hogg et al., 2004). The increase in the lymphocyte population is also associated with bronchial associated lymphatic tissue (BALT) which is hardly seen in normal-lung function non-smokers but frequently seen in smokers (J. C. Hogg, 2004). The existence of lymphocyte and BALT in COPD suggests that the adaptive immune system is involved and might be associated with microbial colonisation and infections of the lower respiratory tract in COPD (J. C. Hogg, 2004; J. C. Hogg & Timens, 2008).

Alveolar attachments to small airways, which provide support during expiration preventing the airways from collapsing, are thought to be damaged as the inflammation progresses. It is still not well understood whether small airway obstruction in COPD is because of bronchiolitis or due to obliteration of surrounding parenchyma and the loss of alveolar attachments, but probably both contribute to small airway obstruction (Szilasi et al., 2006).

2.4 Lung parenchyma component

The parenchymal component of the disease involves the process of emphysema which is further divided into two major types; centrilobular or centriacinar and panlobular or panacinar emphysema.

2.4.1 Emphysema

Emphysema in COPD has been defined as the enlargement of the distal airspaces, beyond the terminal bronchioles, caused by destruction of the alveolar walls (Figure 2.7) ("The definition of emphysema. Report of a National Heart, Lung, and Blood Institute, Division of Lung Diseases workshop," 1985; MacNee, 2005).

Laennec was the first to explain the lesions produced by emphysema characterized by dilation and destruction of the lung parenchyma (J. C. Hogg, 2004; Kligfield, 1981; Laennec, 1834.) leading to a decrease in maximal expiratory airflow by reducing the elastic recoil force available to drive air out of the lungs (J. C. Hogg, 2004; Macnee, 2007).

The two major types of emphysema referred to above (Figure 2.8) are differentiated according to the region of lung acinus being destroyed. Centrilobular or centriacinar emphysema results from destruction and dilation of respiratory bronchioles in the central portions of the acinus. This type of emphysema is usually related to tobacco smoke and is especially severe in the upper lobes of the lung. Panlobular or panacinar emphysema on the other hand involves a more even destruction and dilation over the entire acinus and is usually associated with the lower lobes of the lungs, for example in $\alpha 1$ anti-trypsin deficiency. $\alpha 1$ anti-trypsin acts as anti-elastase to protect the lungs from destruction (J. C. Hogg, 2004; W. D. Kim et al., 1991; M. Saetta, 2006), its genetic absence or low levels may lead to lung destruction at young age and can also lead to liver disease in children (P. Lee, Gildea, & Stoller, 2002).

A diverse variety of processes seems to be involved in the pathogenesis of emphysema related to tobacco smoking, but the most widely accepted hypotheses relate protease-antiprotease or oxidant/anti-oxidants imbalance. The hypothesis states that the destruction of alveolar walls results from protease activity leading to obliteration of extracellular matrix tissue (ECM), but the various proteases involved and their specific targets is still a matter of debate (Abboud & Vimalanathan, 2008; J. C. Hogg, 2004; J. C. Hogg & Senior, 2002).

Even though the protease-antiprotease hypothesis has predominated it still doesn't completely explain the pathogenesis of emphysema because if that was the case then all inflammatory lung diseases should be associated to emphysema (Walters, Reid, Soltani, & Ward, 2008). However, there is growing evidence for the vascular hypothesis which states that reduced vascularity might be the primary defect leading to development of emphysema. In 1959 Liebow reported a scarcity of vessels in the lungs of people with emphysema (Liebow, 1959) and later it was reported that inhibition of VEGF receptors can cause emphysema in rats (Kasahara et al., 2000). The same investigators later demonstrated that the process involved a progressive loss of capillary endothelial cells and epithelial cells due to apoptosis secondary to a decrease in vascular endothelial growth factor (VEGF) and VEGF receptor 2, the active VEGFR (Kasahara et al., 2001). Thus, VEGF might be playing a key role in COPD pathogenesis but unfortunately very little data is available on vascular

changes in human COPD, compared for example to work in asthma (Walters et al., 2008).

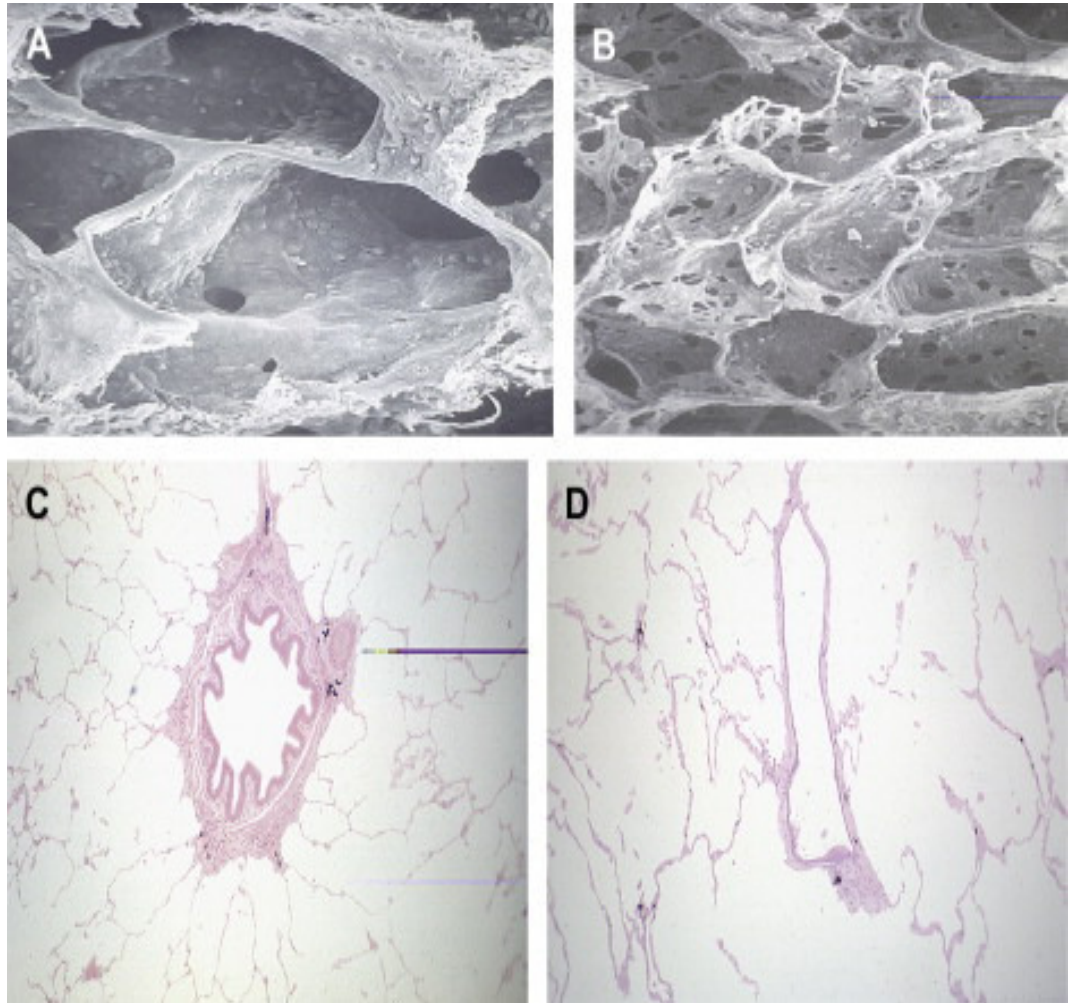


Figure 2.7: Pathology of emphysema.

Scanning electron micrographs of (A) a normal alveoli and (B) early emphysema with holes in alveolar walls. (C) Histologic section of a normal airway with surrounding alveolar attachments. (D) Enlargement of distal airspaces and reduced alveolar attachments in collapsed airway in emphysema (Macnee, 2007).

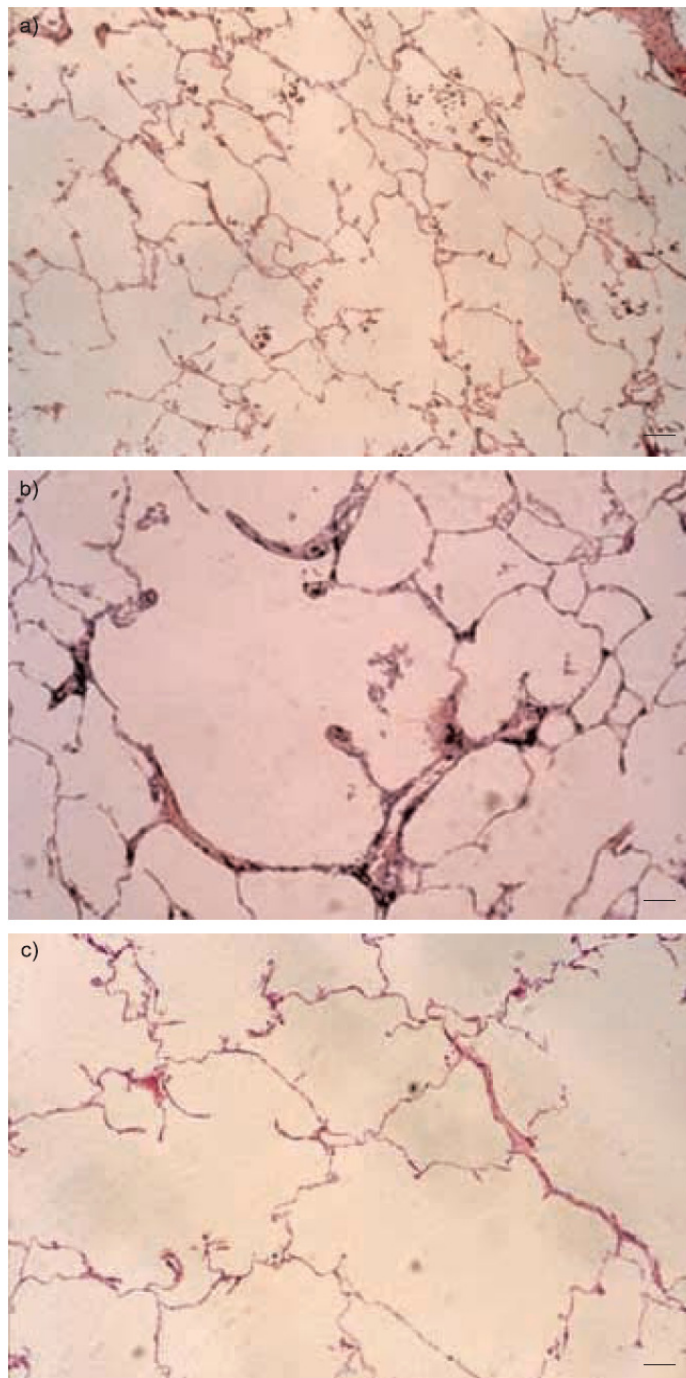


Figure 2.8: Photomicrograph showing the microscopic appearance of the different types of emphysema.

(A) Normal lung; (B) Centriacinar emphysema showing an area of destruction and end enlargement of airspaces around a bronchiole in the central portion of the acinus surrounded by areas of grossly normal lung parenchyma; and (C) panacinar emphysema demonstrating homogenous enlargement of all air spaces beyond the terminal bronchiole (M. Saetta, 2006).

2.5 Airway inflammation in COPD

Inflammation observed in the respiratory tracts (airways and lungs) from patients with COPD is multifaceted and not well understood; even though it involves the activation of both innate and adaptive immune responses. Thus, the inflammatory response observed in COPD is very diverse tautologous and recruitment of various inflammatory cells and mediators contribute significantly to the lung injury in patients with COPD, and appears to serve as a self-perpetuating stimulus for further enhancement of the immune response. The progression of disease involves an active migration of “leukocytes” (mainly neutrophils and macrophages) associated with production of inflammatory mediators and potentially destructive pro-inflammatory cytokines, proteases and various growth factors, which lead to structural changes in the airways termed as “airway remodelling” (explained in detail latter on) (V. Kim, Rogers, & Criner, 2008; Rumora et al., 2008). Chronic inflammation in COPD in response to chronic cigarette smoke and then other exposures, (Figure 2.2) is mainly characterised by accumulation of neutrophils, macrophages, B-cells lymphoid aggregates and CD8+ T-cells, especially in small airways and inflammation becomes worse with disease severity (Sutherland & Martin, 2003).

COPD is predominantly a neutrophilic disease at least in the airway and airspace lumen. Neutrophils are usually found in bronchial epithelium, glands and also in airway smooth muscle bundles but mainly in the airway lumen of both small and large airways, as reported in sputum and BAL (bronchoalveolar lavage) (K. F. Chung & Adcock, 2008). Sietta et al assessed number of neutrophils, eosinophils, mast cells, macrophages, CD4⁺ and CD8⁺ T-lymphocytes, and the ratio of CD4⁺ to CD8⁺ cells in the bronchial glands, epithelium, and lamina propria by immunohistochemistry. They found that smokers with symptoms of chronic bronchitis had an increased number of neutrophils and macrophages and a decreased CD4⁺/CD8⁺ ratio in the bronchial glands when compared to smokers without symptoms, and smokers with chronic bronchitis also had increased number of epithelial neutrophils, whereas the numbers of macrophages and CD4⁺ and CD8⁺ T-lymphocytes in the epithelium and lamina propria were similar in the two groups of smokers (Sietta et al., 1997). In another study it was shown that current smokers with COPD had increased number of neutrophils and CD8+ T cells in the airway

smooth muscle of peripheral airways compared with non-smokers, and they also reported that smokers with normal lung function also had a neutrophilic infiltration in the airway smooth muscle, but to a lesser extent compared to current smokers with COPD (Baraldo et al., 2004). When all the groups were analysed as one group neutrophilic infiltration in the smooth muscle of peripheral airways inversely related to FEV1% predicted (forced expiratory volume in one second) (Baraldo et al., 2004). Stanescu et al reported increased levels of neutrophils in sputum of smokers and showed that airways obstruction, chronic expectoration, and rapid decline of FEV1 in smokers are associated with increased levels of sputum neutrophils (Stanescu et al., 1996).

Different studies have variably reported increased levels of eosinophils in the airway wall and BAL and also in induced sputum, but the role of eosinophils in COPD are not very clear. It is suggested that eosinophilia may represent a different sub-group of COPD (K. F. Chung & Adcock, 2008). Interestingly, a few studies have reported that an increase in BAL and sputum eosinophils in COPD patients is related to good clinical response to steroid therapy (Chanez et al., 1997; Fujimoto, Kubo, Yamamoto, Yamaguchi, & Matsuzawa, 1999; Pizzichini et al., 1998), but this was not replicated by our group who could also not relate eosinophils to bronchodilator responsiveness in COPD (D. W. Reid et al., 2008).

The situation of mast cells in COPD is not very clear either, with some studies reporting an increase in airway mast cells while others not. Pesci et al investigated BAL and bronchial biopsies with immunohistochemical techniques for mast cells in current smokers and ex-smokers with chronic bronchitis, and found that subjects with “chronic bronchitis” (both current and ex-smokers with cough and sputum) had higher numbers of mast cells both in the epithelium and in the bronchial glands compared to normals, whereas the numbers of mast cells in BAL and in the lamina propria were similar in the two groups. In current smokers with bronchitis an increase in mast cell numbers was observed in the epithelium and lamina propria, and in BAL compared to ex- smoker bronchitis (Pesci et al., 1994). In another study, it was reported in small airway samples from COPD patients (both current smokers and ex-smokers) that numbers of epithelial mast cells and macrophages increased in the airway wall of smokers with airflow limitation, suggesting a potential role for mast

cells in development of COPD (Grashoff et al., 1997). On the other hand, Saetta et al reported no change in mast cell numbers in the bronchial mucosa of subjects with chronic bronchitis (Saetta et al., 1993). The same group later reported normal mast cell numbers in current smokers with COPD and also in normal lung function smokers (Di Stefano et al., 1998). In a similar study, Shaughnessy et al reported no change in sub-epithelial mast cells in subjects with chronic bronchitis (O'Shaughnessy, Ansari, Barnes, & Jeffery, 1997).

More consistently, CD8+ T cells are reported increased in COPD airways and also in the lung parenchyma (Peter J. Barnes, 2008). Shaughnessy et al reported that the number of CD8+ T cells increase in the sub-epithelial zone (lamina propria) of bronchial biopsies from patients with chronic bronchitis and inversely correlated to FEV1 (O'Shaughnessy et al., 1997). Baraldo et al reported increased numbers of CD8+ T cells localised within the smooth muscle of peripheral airways of COPD patients (Baraldo et al., 2004). Chrysafakis et al also reported increased levels of CD8+ T cells in sputum of smokers with and without COPD compared to non-smokers, and interestingly they also reported that they are highly active and expressed high levels of lytic substances such as perforin which may lead to parenchymal damage in COPD lung (Chrysafakis et al., 2004). Saetta et al also reported increased CD8+ T cells in peripheral airway wall of smokers who develop COPD, suggesting potential role in COPD pathogenesis (Saetta et al., 1998).

Gosman et al reported increased number of B-cells in the sup-epithelial region of bronchial biopsies from COPD patients (Gosman et al., 2006). Severe airway obstruction is associated with presence of B cells, organized into follicles in small airways (James C. Hogg et al., 2004). As also described earlier, increase in the lymphocyte population is also associated with bronchial-associated lymphatic tissue (BALT) which is hardly seen in physiologically normal non-smokers but frequently seen in smokers (J. C. Hogg, 2004). The existence of lymphocytes and BALT in COPD suggests that the adaptive immune system is involved and might be associated with microbial colonisation and infections of the lower respiratory tract in COPD (J. C. Hogg, 2004; J. C. Hogg & Timens, 2008). Dendritic cells along with B-cells have also been reported to play a role in adaptive immunity in COPD (K. F. Chung & Adcock, 2008). Van der et al reported lymphoid follicles consisting of B cells and

follicular dendritic cells with adjacent T cells both in the parenchyma and in bronchial walls of patients with emphysema (van der Strate et al., 2006). Demedts et al reported increased numbers of dendritic cells in the epithelium of small airways of patients with COPD compared with never-smokers and smokers without COPD (Demedts et al., 2007). Additional evidence for recruitment of dendritic cells by tobacco smoke exposure comes from studies done in mice in which chronic cigarette exposure led to an increase in CD11c+ dendritic cells (D'Hulst A et al., 2005).

It is suggested that COPD may also have an auto-immune component as it is a chronic inflammatory disease, albeit with a much better recognised aetiology than other such illness e.g. rheumatoid arthritis (RA). But like RA, once initiated, the inflammatory response COPD seems to be self-perpetuating even after smoking cessation (Agusti, MacNee, Donaldson, & Cosio, 2003). In a very interesting study, Lee et al showed that emphysema is an autoimmune disease, which could be characterised by circulating anti-elastin antibodies and Th1-type immune responses, which correlated with disease severity (Peter J. Barnes, 2008; S. H. Lee et al., 2007). Feghal et al reported increased levels of IgG auto-antibodies with increased avidity for pulmonary epithelium, and the potential to mediate cytotoxicity in patients with COPD (by using immuno-fluorescence and immuno-precipitation and immuno-histochemistry techniques), suggesting that auto-reactive adaptive immune responses may be important in the etiology of COPD (Feghali-Bostwick et al., 2008). The jury is still out on whether these observations are centrally important, or whether they just represent an epi-phenomenon related to release of airway antigens by tissue proteolytic damage.

Macrophage numbers are increased in COPD, to a greater extent compared to asthma (Peter J. Barnes, 2008). Saetta et al observed increased numbers of CD68+ macrophages in the sub-epithelial lamina propria of patients with chronic bronchitis (Saetta et al., 1993). In another study clusters of macrophages were observed in small airways associated with peri-bronchial fibrosis seen in smokers and ex-smokers and it was suggested that they may be related to small airway fibrosis and/or development of centrilobular emphysema (Fraig, Shreesha, Savici, & Katzenstein, 2002). Finlay et al showed that macrophages from BAL of patients with emphysema express higher levels of mRNA for MMP-1 and MMP-9 (matrix metalloproteinases),

and they demonstrated that alveolar macrophages from the emphysematous lung produced large amounts of matrix-degrading enzymes which may play an important role in development of COPD (Finlay et al., 1997). Others have also suggested that alveolar macrophages could be orchestrators of COPD (Barnes, 2004).

2.6 Airway remodelling in COPD

Airway inflammation is the response of a fully vascularised tissue to injury and the rationale behind the inflammatory response is to protect the host and to restore normal tissue functioning. It is widely assumed that accelerated decline in lung function in COPD is a consequence of acute inflammation leading to parenchymal and airway wall remodelling respectively. However, whether remodelling is part of the inflammatory process, or whether it is a separate and parallel entity is still a matter of debate and there is no convincing evidence either way available in the literature (P. K. Jeffery, 2001b). In reality there are very few data available on airway remodelling in COPD, even in comparison to asthma where many questions remain to be answered.

During lung development, the lung undergoes extensive “modelling” and then “remodelling” changes and this whole process is very tightly regulated by different signalling pathways, growth factors and various cytokines. It may be possible that the perception of remodelling in airway disease is a reactivation of these embryological mechanisms as most of the cytokines and growth factors thought to be detrimental in COPD and other respiratory disorders are expressed normally during lung morphogenesis (P. K. Jeffery, 2001b; Warburton et al., 2001).

Currently airway remodelling is defined as an alteration in size, mass, or number of tissue structural components leading to gross thickening of the airway wall occurring in response to oxidant or other injury and/or inflammation (P. K. Jeffery, 2001b). In COPD, remodelling may occur as a response to smoking-induced damage to the airways, but the mechanisms of damage and initiation, and even the structural changes themselves are poorly described compared to work published in asthma (Churg et al., 2006; Saetta et al., 2000). Structural changes mainly described in COPD are: small airway wall thickness (James C. Hogg et al., 2004), metaplasia of

the epithelium, loss of epithelial cilia, increase in goblet cell size and number, submucosal gland and smooth muscle hypertrophy (Figure 2.9 & 2.10) (Bergeron & Boulet, 2006; Saetta et al., 2000).

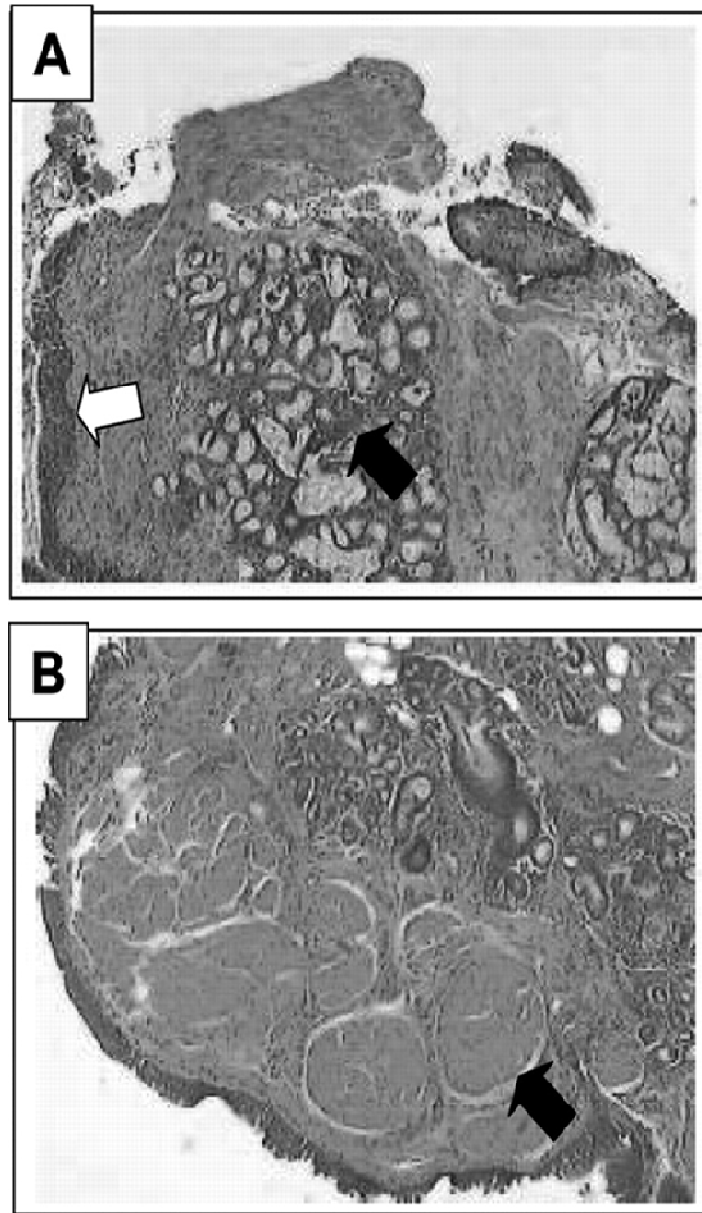


Figure 2.9: Airway structural changes in COPD.

(A) Epithelial metaplasia (white arrow) and submucosal gland hyperplasia (black arrow). (B) Increase in smooth-muscle mass (black arrow) (Bergeron & Boulet, 2006).

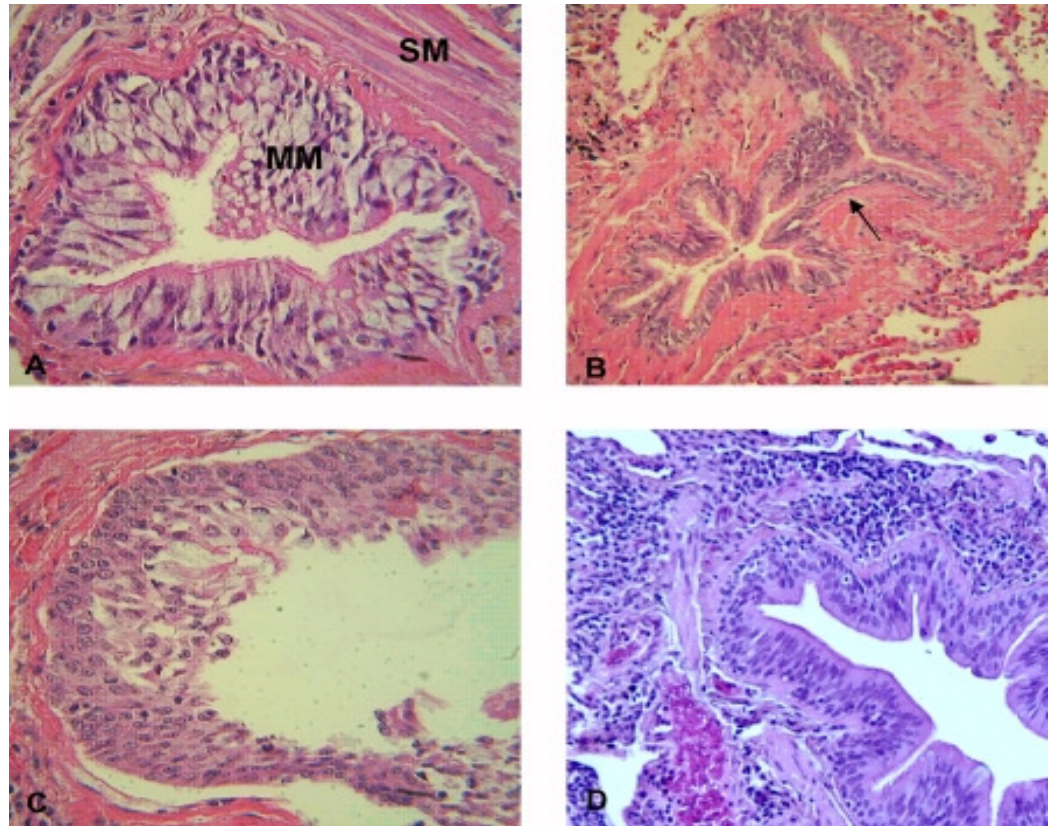


Figure 2.10: Examples of airway remodelling in COPD. (A) Represents mucous metaplasia (MM) of the epithelium and smooth muscle hypertrophy (SM). (B) Represents peribronchial fibrosis (*black arrow*). (C) Shows squamous metaplasia. (D) shows an inflammatory infiltrate of lymphocytes in the adventitia of a bronchiole (V. Kim et al., 2008).

2.6.1 Epithelium

Respiratory epithelium is described as pseudostratified “columnar”, consisting of eight different types of epithelial cells classified into three main categories: basal, ciliated and secretory. It is a highly active part of the airways and acts as a chemical, physical, and immunological barrier. It is also the site of first contact for cigarette smoke and plays an important role in protecting the lung (Davies, 2009; Knight & Holgate, 2003; Thorley & Tetley, 2007).

Epithelial shedding is not widely reported in COPD; however denuded epithelium has been reported in young smokers in small airways associated with increased inflammatory cell infiltration (M. G. Cosio, Hale, & Niewoehner, 1980). Thick epithelium, loss of cilia, goblet cell hyperplasia and squamous metaplasia (transformation of a columnar epithelium into a squamous epithelium) are also frequently observed and reported in COPD (Figure 2.9 & 2.10) (P. K. Jeffery, 2001b; V. Kim et al., 2008).

Chronic exposure to cigarette smoke activates various oxidative pathways leading to the secretion of a diverse variety of cytokines and chemokines and various epithelial derived growth factors including; granulocyte- macrophage colony stimulating factor (GM-CSF), which is secreted by a wide variety of cells in the airways including epithelium, airway smooth muscle, fibroblasts, T lymphocytes, mast cells, eosinophils and macrophages (Saha et al., 2009). These can lead to alterations in the reticular basement membrane (Rbm) by activating fibroblasts/myofibroblasts, sub-mucosal glands, smooth muscle and the vascular bed (P. K. Jeffery, 2001b). However, few studies have shown potential for GM-CSF in COPD, and no direct evidence is available (Saha et al., 2009).

EGFR expression and function has been studied broadly in asthma and reported in several studies, but fewer data are available in COPD. Cigarette smoke has the potential to activate signaling associated with EGFR which can further lead to mucus hypersecretion (Shao, Nakanaga, & Nadel, 2004). EGFR can induce abnormal epithelial cell growth, differentiation, migration and proliferation leading to goblet cell hyperplasia and hence to mucus hypersecretion in COPD (de Boer et al., 2006; Innes et al., 2006).

Transforming growth factor- β 1 (TGF- β 1) expression has been observed in the epithelium in bronchial biopsies from COPD patients (Kokturk, Tatlicioglu, Memis, Akyurek, & Akyol, 2003) and is also considered as one of the major growth factors driving the process of airway remodelling generally in inflammatory airway disease. Some studies have reported that interactions between TGF- β and its second messenger, Smad signalling pathway play an important role in airway remodelling

by regulating extracellular matrix (ECM) turnover (Figure 2.11) (Postma & Timens, 2006). Springer and colleagues demonstrated that Smad signalling is altered in COPD due to a decrease in TGF- β inhibitory Smad-6 and Smad-7 observed in epithelium in bronchial biopsies from COPD patients compared to normal controls. This may suggest that inhibitory pathways are distorted in COPD leading to lack of control of fibrotic changes (Springer, Scholz, Peiser, Groneberg, & Fischer, 2004).

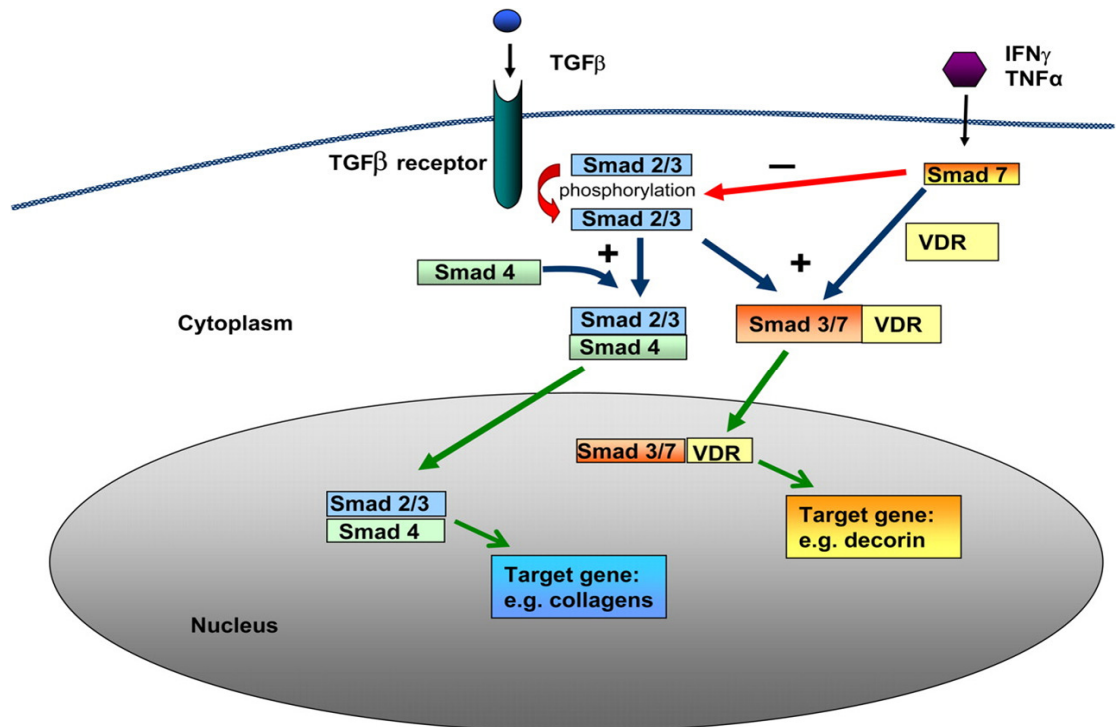


Figure 2.11: Main components of the Smad pathway (Gosman et al., 2006).

Increased vascular endothelial growth factor (VEGF) expression in the epithelium has also been reported in COPD compared to normal non-smokers, which may suggest a role for VEGF in epithelial repair in response to cigarette smoke exposure (Kranenburg, de Boer, Alagappan, Sterk, & Sharma, 2005). The exact role of VEGF in epithelium is not clear but a significant body of literature suggests a decrease in VEGF in lung parenchyma may be associated with emphysema (Liebow, 1959).

Cigarette smoke increases the oxidative stress in the lungs which also leads to increased proteolytic load in the lungs of smokers through infiltrating neutrophils and macrophages, but there is very convincing evidence available now that the epithelium itself has the capacity to secrete a variety of proteases, mainly matrix-metalloproteinases (MMPs). MMPs are the largest family of proteases with more than 25 members identified so far. They have the capacity to degrade all the components of the ECM, (Thorley & Tetley, 2007) playing a key role in matrix turnover, remodelling and angiogenesis (Kian Fan Chung, 2005).

Immunohistochemical studies (Baraldo et al., 2007; Thorley & Tetley, 2007) and sputum profiles (Mercer et al., 2005) in COPD have shown that MMP-2 and MMP-9 levels are elevated during disease progression. On the other hand it was also shown that epithelial anti-proteases like SERPIN2 (serine protease inhibitor) which codes for a thrombin and urokinase inhibitor (Demeo et al., 2006), tissue inhibitors of metalloproteinases TIMPs (Mercer et al., 2005) and secretory leukocyte protease inhibitors (SLPI) (Thorley & Tetley, 2007) are elevated during chronic inflammation in COPD. This suggests activation of defensive mechanism of the epithelium against the enhanced proteolytic activity induced by smoking. It is the balance of this “combat” between competing proteolytic forces that may decide the fate of the airway and lung in smokers.

2.6.2 Reticular basement membrane (Rbm)

The human airway epithelium is attached to a true basement membrane, which further consists of a lamina rara and a lamina densa. The reticular basement membrane, also known as lamina reticularis, is located just below the true basement membrane extending superficially (Postma & Timens, 2006; Saglani et al., 2006), and separates the epithelium from the underlying lamina propria mesenchymal structure.

Immunohistochemical studies have confirmed that true basement membrane is comprised mainly of type IV collagen (Montes, 1996) and is attached strongly to the underlying Rbm by collagen VII strands (Liesker et al., 2009). In asthmatics Rbm is homogenous and hyaline in appearance (Peter K. Jeffery, 2004) (Figure 2.12) and

comprises mainly collagen I, III, V, fibronectin, tenascin (Postma & Timens, 2006) and laminin (Liesker et al., 2009) and is thickened (P. K. Jeffery, 2001b). Thickening of the Rbm in asthma has a strong positive correlation with the number of subepithelial fibroblasts and myofibroblasts (Brewster et al., 1990). Rbm develops in normal healthy individuals during childhood, but thickening may develop early in individuals with asthma perhaps even before symptoms start (P. K. Jeffery, 2001b).

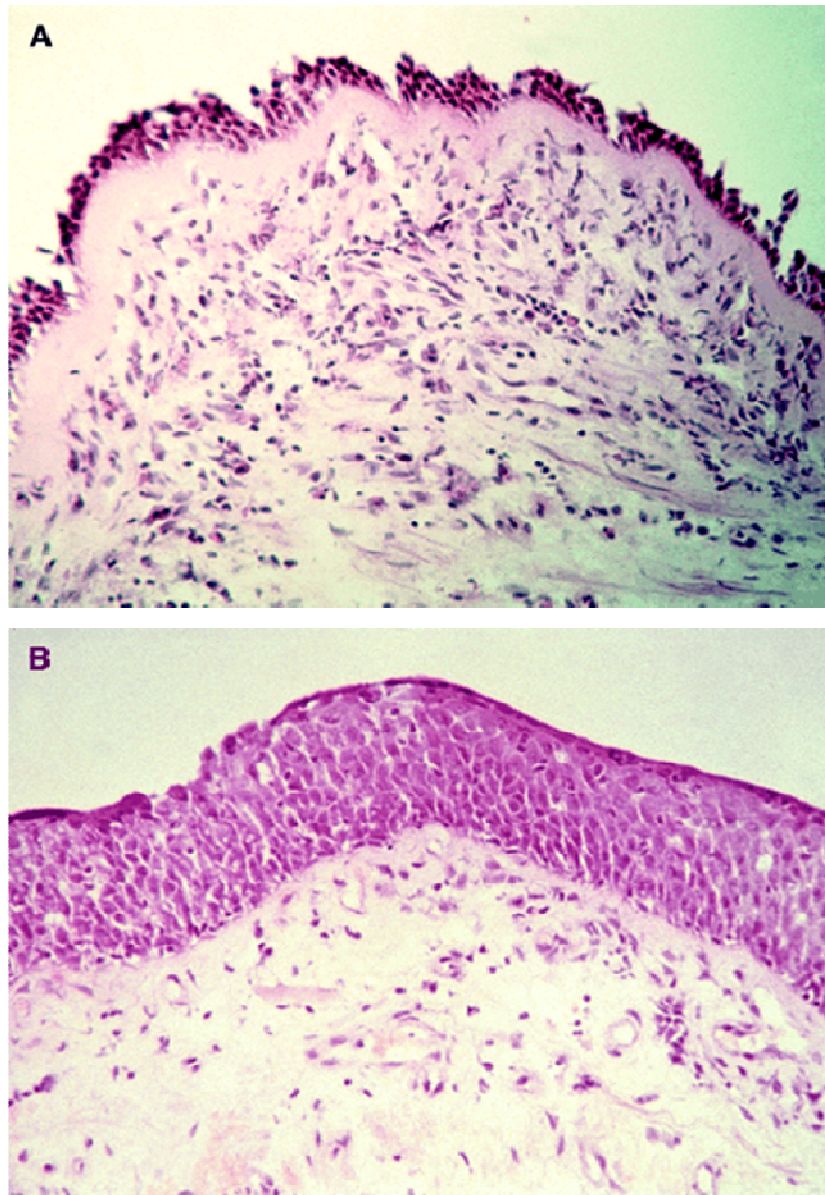


Figure 2.12: The airway mucosa as it appears by light microscopy (LM) of bronchial biopsies from (A) an atopic asthmatic showing loss of surface epithelium and early thickening and hyaline appearance of the reticular basement membrane Rbm and (B) a heavy smoker with COPD demonstrating epithelial squamous metaplasia and a thinner Rbm of normal thickness. Stained with haematoxylin-eosin (H&E) (P. K. Jeffery, 2001b).

In COPD, data on Rbm are rare and very ambiguous; there is no clear evidence on Rbm thickness, composition or appearance (Figure 2.12), although some studies have reported that tenascin is increased in COPD Rbm and there is also a trend towards stronger type IV collagen staining. Less collagen I and laminin is also reported in COPD Rbm compared to asthma (Kranenburg et al., 2006; Liesker et al., 2009). Rbm thickness in COPD is still a matter of debate, with some studies showing increased thickness while others report no difference (Liesker et al., 2009), but it appears that composition of reticular basement membrane is different in COPD subjects compared to asthmatics (Liesker et al., 2009; Postma & Timens, 2006).

Interstitial collagen (forming scar tissue), which lies deeper than the Rbm and reported as airway wall fibrosis, is usually considered as the feature of smokers who are more likely to develop COPD (especially in small airways), but no such evidence of fibrosis is available in large airways (Bosken, Wiggs, Pare, & Hogg, 1990; Peter K. Jeffery, 2004).

2.6.3 Extracellular matrix

Increase in airway wall tissue has been observed due to increase in volume of epithelium, lamina propria, muscle and adventitial compartments of small airways and may lead to fixed airflow limitation in COPD (James C. Hogg et al., 2004). In asthma matrix deposition is mainly observed in the subepithelium Rbm area but in COPD the whole airway wall seems to be affected. However, there are actually very few studies reporting matrix changes and their exact role in COPD, this needs further investigations (Kian Fan Chung, 2005), which we aim to do.

Van Straaten et al reported immunohistochemical studies in lung tissues from patients with mild and severe emphysema, and from patients with lung fibrosis. They looked at collagens, laminin, fibronectin, proteoglycans and beta1-integrins and reported that majority of the patients with severe emphysema showed a decrease in staining for the interstitial proteoglycans decorin and biglycan in the peribronchiolar area, compared to controls and patients with fibrosis. Very few patients with mild emphysema showed this pattern of reduced staining. In comparison, decorin and biglycan were strongly positive in the perivascular area of all of the sections from

patients with emphysema. There was no change to collagen type I, III and IV and also no abnormality was observed in laminin or fibronectin expression in the three pathological groups. They also suggested that specific loss of interstitial proteoglycans may be essential for elastic recoil loss and following bronchiolar obstruction, as seen in patients with smoking-related emphysema (van Straaten et al., 1999).

Somewhat contradictory to these findings, Kranenburg et al (Kranenburg et al., 2006) in another immunohistochemical study observed that COPD has increased expression of collagen type I, III and IV, fibronectin and laminin and also increased expression of matrix proteins in the Rbm, lamina propria and adventitia of the bronchial walls and vessels. At the site of damaged bronchial epithelium in the surface epithelial basement membrane, enhanced matrix protein deposition was observed. They also observed that collagens I and III were dominant in COPD patients in the reticular layer near the lamina propria, when compared to normal controls, and there was also increased expression of fibronectin and laminin observed in bronchial vessels. FEV1 correlated inversely with the collagen in the Rbm region, fibronectin in bronchial vessels and laminin in the airway smooth muscle layer. These findings may indicate a pathogenic role for matrix proteins in airway remodelling in COPD, but these studies are limited by the fact that tissue was obtained from COPD patients who had undergone surgery for lung cancer. There is no direct evidence from airway biopsies in otherwise healthy non-cancer COPD patients (Kranenburg et al., 2006).

2.6.4 Vascularity

The literature on vascular remodelling and angiogenesis in chronic airway disease is scant, although there are studies reported in asthma (Feltis et al., 2006; McDonald, 2001). Vascular remodelling involves structural changes, usually enlargement of arterioles, capillaries or venules without the formation of new vessels whereas angiogenesis is the growth of new blood vessels from the existing ones (Feltis et al., 2006; McDonald, 2001).

Dunnill et al in 1960 (Dunnill, 1960) were the first to describe enlargement of the capillary bed in the airway wall of patients who died of chronic asthma. Increased

vascularity was later reported in mild asthmatics as well, but perhaps especially in severe corticosteroid dependent asthmatics (Peter K. Jeffery, 2004). Bronchial mucosal blood vessel dilation, congestion and wall edema are reported as hallmarks of chronic asthma and may account for substantial swelling and stiffening of the airway wall (Peter K. Jeffery, 2004). In another study, Khor, et al suggested that airway vascular leakage is a major pathophysiologic feature of early asthma deterioration, occurring before recrudescence of cellular inflammation (Khor et al., 2009).

Several angiogenic growth factor cytokines have been recognized including members of the fibroblast growth factor (FGF) family, vascular endothelial growth factor VEGF, TGF- α and TGF- β , angiogenin, platelet derived growth factor, tumor necrosis factor- α (TNF- α), GM-CSF, hepatocyte growth factor (HGF), and various interleukins and chemokines such as IL-8. Angiopoietin 1 and 2 are also involved (Postma & Timens, 2006; Puxeddu, Ribatti, Crivellato, & Levi-Schaffer, 2005), perhaps more in a vessel stabilisation/maturation role. Among all the different angiogenic factors, VEGF has been recognised as the key growth factor involved in regulation of angiogenesis (Figure 2.13) and is highly expressed during chronic inflammation (Postma & Timens, 2006).

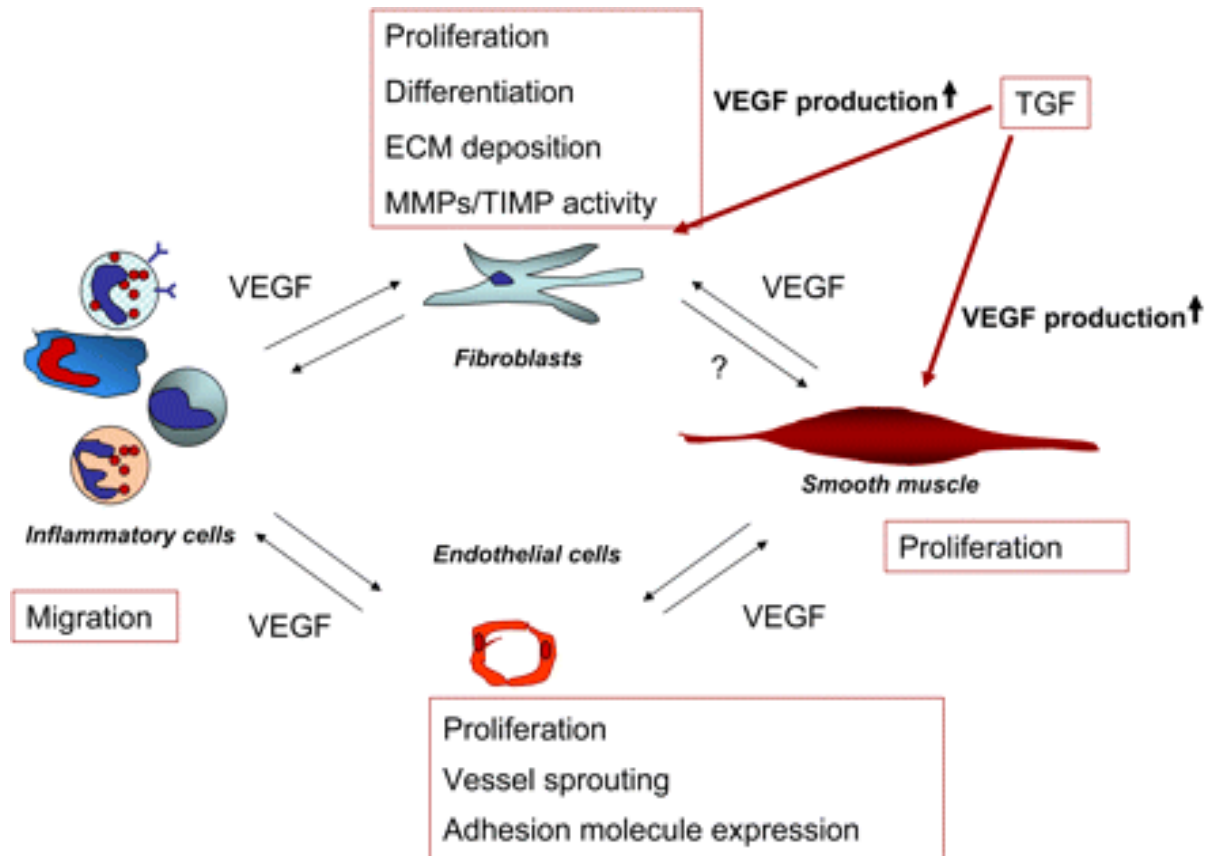


Figure 2.13: Schematic representation of the main regulatory roles of vascular endothelial growth factor (VEGF). ECM = extracellular matrix; TGF = transforming growth factor; MMP = matrix metalloproteinase; TIMP = tissue inhibitor of metalloproteinase (Gosman et al., 2006).

Imbalance between VEGF and endostatin (a potent anti-angiogenic factor) levels has been observed in the sputum of patients with asthma, this suggests that this disparity may trigger proangiogenic factors leading to abnormal new blood vessel formation in asthma (Asai et al., 2002). Hoshino and colleagues reported that VEGF expression increases in the airway mucosa of patients with asthma compared to normal controls (Hoshino, Nakamura, & Hamid, 2001). A very interesting study reported higher levels of VEGF in bronchoalveolar lavage fluid and in bronchial biopsies in subjects with asthma which were related to number of vessels (Feltis et al., 2006). Angiogenic sprouts (i.e., early-forming vascular structures) were also observed and their number increased in subjects with asthma (Feltis et al., 2006).

Yet again, data on COPD are much more limited. Vascular abnormalities have been reported in COPD, and associated with disease progression (Postma & Timens, 2006). Kranenburg and colleagues reported enhanced expression of VEGF in the bronchial, bronchiolar and alveolar epithelium and macrophages. They also observed VEGF expression in airway smooth muscle and vascular smooth muscle cells in both the bronchiolar and alveolar compartments (Kranenburg et al., 2005). They suggested that VEGF and its receptors VEGFR1 (decoy) and VEGFR2 (active) may be involved in epithelial and endothelial cell repair and maintenance in response to injury caused by cigarette smoking and may be associated with airway remodelling in COPD (Kranenburg et al., 2005; Siafakas, Antoniou, & Tzortzaki, 2007).

In a very interesting study, Calabrese and colleagues (Calabrese et al., 2006) evaluated the contribution of vascularity in airway remodelling in smokers with normal lung function and smokers with COPD. They performed an immunohistochemical analysis involving vessels positive for integrin $\alpha v \beta 3$. High $\alpha v \beta 3$ expression was observed in bronchial vessels which was associated with higher cellular expression of VEGF, suggesting that these two molecules might be playing an important interacting role in angiogenesis. Enhanced bronchial vascularity was also observed as both number of vessels and vascular area of lamina propria in smokers with normal lung function and smokers with COPD compared to normal healthy controls. These data from normal lung function smokers may suggest that

vascular changes might be independent of the airway obstruction and severity of the disease (Calabrese et al., 2006).

Inflammation, which is one of the hallmarks of COPD, also has the potential to promote angiogenesis in numerous ways (Figure 2.14) (Siafakas et al., 2007). Airway inflammation in COPD mainly involves infiltration of neutrophils, macrophages and CD8+ T cells in the lumen, bronchial and bronchiolar airway wall and lung parenchyma (P. K. Jeffery, 2001a; Siafakas et al., 2007). These inflammatory cells in the lungs can release various mediators such as TNF- α which can promote angiogenesis. Hypoxia can also occur in COPD either globally or focally in inflamed tissue, which may also induce angiogenesis (Siafakas et al., 2007).

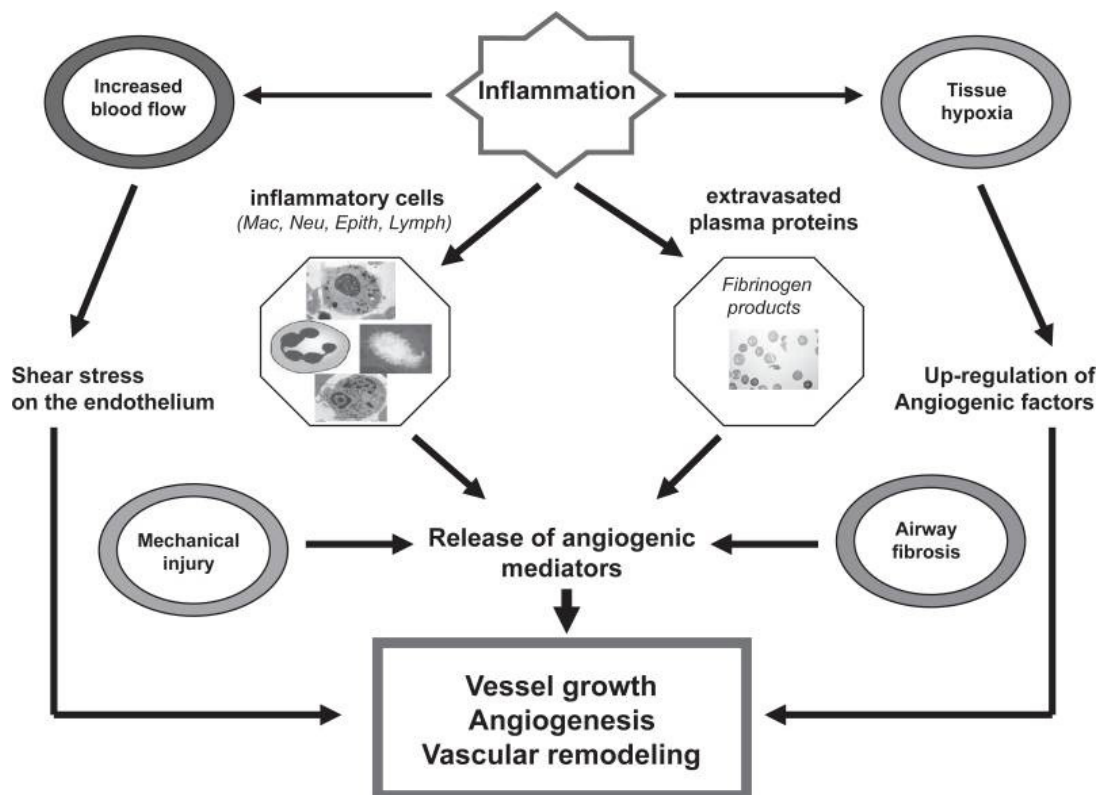


Figure 2.14: Schematic representation of an angiogenic process in COPD (Siafakas et al., 2007).

Hypoxia has also been considered a key factor involved in pulmonary vascular remodelling leading to pulmonary hypertension in COPD (Nilsson, Shibuya, & Wennstrom, 2004; Wright et al., 1983). However, vascular remodelling of pulmonary arteries is not only the characteristic of severe COPD but has also been reported in patients with mild COPD without arterial hypoxemia and also in smokers with normal lung function (Barbera et al., 1994). It is reported that VEGF could play a major role in remodelling of pulmonary arteries in smokers and COPD (Peinado et al., 1999; Siafakas et al., 2007).

2.6.5 Airway smooth muscle

Bronchial smooth muscle mass (Figure 2.10) and its contribution to airway wall remodelling has been predominantly studied in small airways compared to large airways in COPD (P. K. Jeffery, 2001b). Whether airway smooth muscle mass is a prominent cause of airflow limitation, is still a matter of debate, with no convincing evidence available (V. Kim et al., 2008).

However, an increase in airway smooth muscle in small airways was inversely correlated with lung function in one study (Kian Fan Chung, 2005). In another it was reported that muscle mass was increased by 50% in patients with severe COPD in small airways (James C. Hogg et al., 2004). There is little information available on airway smooth muscle cells, but they may be functionally altered in proximal airways in COPD. Unfortunately there is no information available on function of the airway smooth muscle in small airways. It is not clear whether any increase in muscle mass in COPD is due to an increase in number of muscle cells, or increase in airway smooth muscle size, or a combination of both. In asthma, airway smooth muscle predominantly increases in large airways whereas in COPD this seems to occur mainly in small airways (Kian Fan Chung, 2005).

Abnormalities associated with smooth muscle mass in large airways have not been reported in COPD, although internal airway wall thickness has been associated with reduced FEV1/FVC ratio (Kian Fan Chung, 2005; Peter K. Jeffery, 2004). Even biopsy studies from large airways reported no increase in smooth muscle area and size; moreover, smooth muscle protein isoforms were not increased, but there was a

slight increase in myosin light chain kinase but with no increase in phosphorylated myosin light chain. Thus, more data are required on airway smooth muscle remodelling in large airways in COPD (Kian Fan Chung, 2005).

2.6.6 Goblet cells, submucosal glands and mucus

Goblet cells are mucus-producing cells which are dispersed widely in the airways, reproductive tract and alimentary canal. In the airway their major function is to hydrate, lubricate and to clear the particulate matter and pathogens from the lumen by secreting mucus, which forms a slimy film covering mainly the luminal surface of epithelial cells. Goblets cells are the major secretory cells of the airways and the major source of luminal mucus (Davis & Dickey, 2008; Yoshida & Tuder, 2007).

Goblet cell hyperplasia has been associated with both asthma and chronic bronchitis in large airways (P. K. Jeffery, 2001b). Their number is usually less in small airways, but an increase in peripheral airways (diameter < 1 mm) in COPD subjects has been associated with an influx of neutrophils into the airways promoting the concept that neutrophils might be playing a role in goblet cell degranulation through release of secretagogues neutrophils elastase and cathepsin G (Sommerhoff, Nadel, Basbaum, & Caughey, 1990). In another study done by M. Saetta, et al there was an increase in the number of goblet cells and inflammatory cells in the epithelium of peripheral airways of smokers with chronic bronchitis and airflow limitation (Saetta et al., 2000).

Submucosal gland hypertrophy was described by Reid in 1954 (L. M. Reid, 1954). Mucus gland hypertrophy is observed in both asthma and COPD. In COPD submucosal gland hypertrophy is mainly seen in large airways (P. K. Jeffery, 2001b). Glands are composed of serous and mucus secretory units or acini. In asthma normal proportions of mucus and serous units are retained, whereas in chronic bronchitis this balance is disturbed and there is a disproportionate increase in mucous units and decrease in serous units (Glynn & Michaels, 1960). Serous units in the submucosal glands secrete a wide range of antibacterial substances such as the secretory component of secretory IgA, lysozyme and lactoferrin. Loss of these substances during airway remodelling may create a potential for chronic bacterial colonisation in

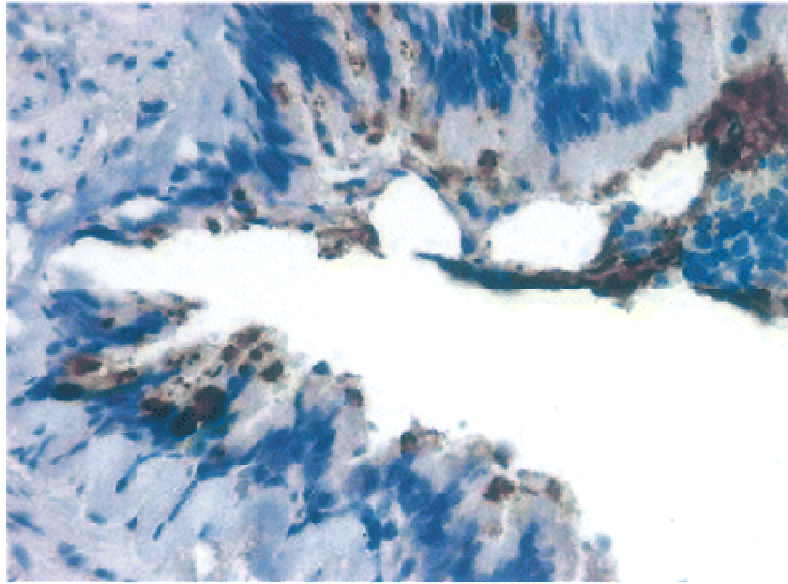
the airways (P. K. Jeffery, 2001b), but interestingly, mucus production and gland hypertrophy are poorly related to each other, whereas inflammation has been shown to be closely associated with mucus hypersecretion (Barbera et al., 1994).

There are very few studies in the literature reporting the composition of mucus in COPD and the various types of mucins involved (Kian Fan Chung, 2005) and data regarding mucus hypersecretion are contradictory. Innes and colleagues reported that epithelial mucin stores are elevated in the large airways of habitual smokers with airflow limitation, with predominant increase in MUC5AC gene expression (gene which encodes for mucin-5AC protein in humans, also commonly known as MUC5AC in literature). They reported significant decrease in MUC5B gene expression (gene which encodes for mucin-5B protein in humans, also commonly known as MUC5B in literature) while other authors found no change (Innes et al., 2006). In peripheral airways Caramori and colleagues found increased expression of MUC5B in the bronchiolar lumen and increases in MUC5AC expression in the bronchiolar epithelium of patients with COPD (Figure 2.15) (Caramori et al., 2004).

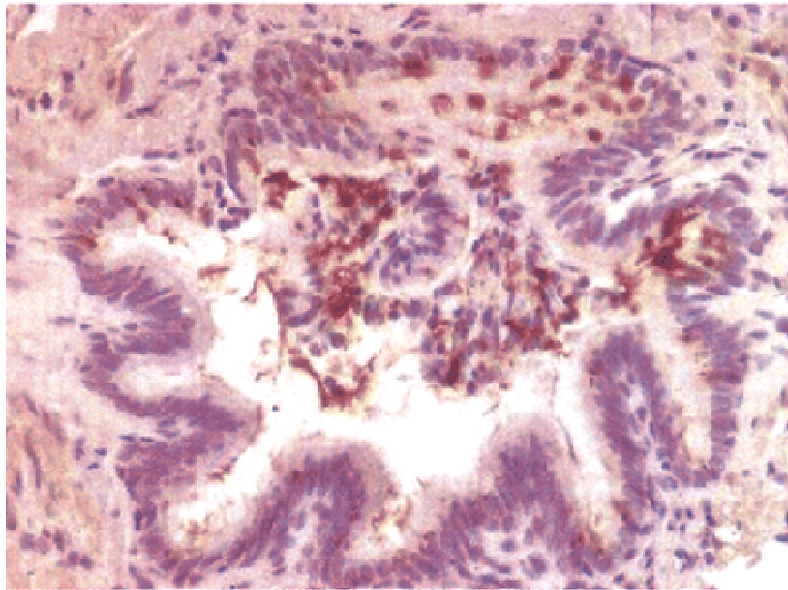
How mucus secretion is controlled is not well understood. However, limited studies have reported that cellular signaling initiated by epidermal growth factor receptor (EGFR) may be playing a key role in mucus production and regulation (Yoshida & Tuder, 2007). Cigarette smoke is considered as the main etiological factor associated with COPD and in humans chronic exposure to tobacco smoke may activate EGFR signalling pathways leading to mucus hypersecretion (Yoshida & Tuder, 2007). EGFR can be activated in two different ways ie, involving a ligand-dependent or a ligand-independent mechanism (Figure 2.16) (Burgel & Nadel, 2004). In ligand-dependent EGFR tyrosine phosphorylation, EGFR ligands bind to their receptor and activate it, but on the other hand in a ligand independent mechanism EGFR tyrosine phosphorylation can occur directly in response to increase in oxidative stress due to cigarette smoke and inflammation (Burgel & Nadel, 2004).

In COPD there are few studies reporting EGFR expression. Immunohistochemical analysis done by De Boer et al revealed that EGFR expression is increased in biopsies from COPD patients (de Boer et al., 2006). EGFR family has four different types of receptors termed ErbB1, ErbB2, ErbB3 and ErbB4 and EGFR itself is also

known as ErbB1. Donnell and colleagues reported enhanced expression of EGFR, ErbB3 and MUC5AC in the airways of long term current smokers; they also observed that this increase was not associated with neutrophilic inflammation and suggested that ErbB3, may be playing an important role in mucus hypersecretion (O'Donnell et al., 2004).



(A) MUC5AC



(B) MUC5B

Figure 2.15: Representative photomicrographs of **(A)** bronchiolar epithelium immunostained for MUC5AC (brown) and **(B)** bronchiolar lumen immunostained for MUC5B (brown) from smokers with chronic obstructive pulmonary disease (COPD) (Caramori et al., 2004).

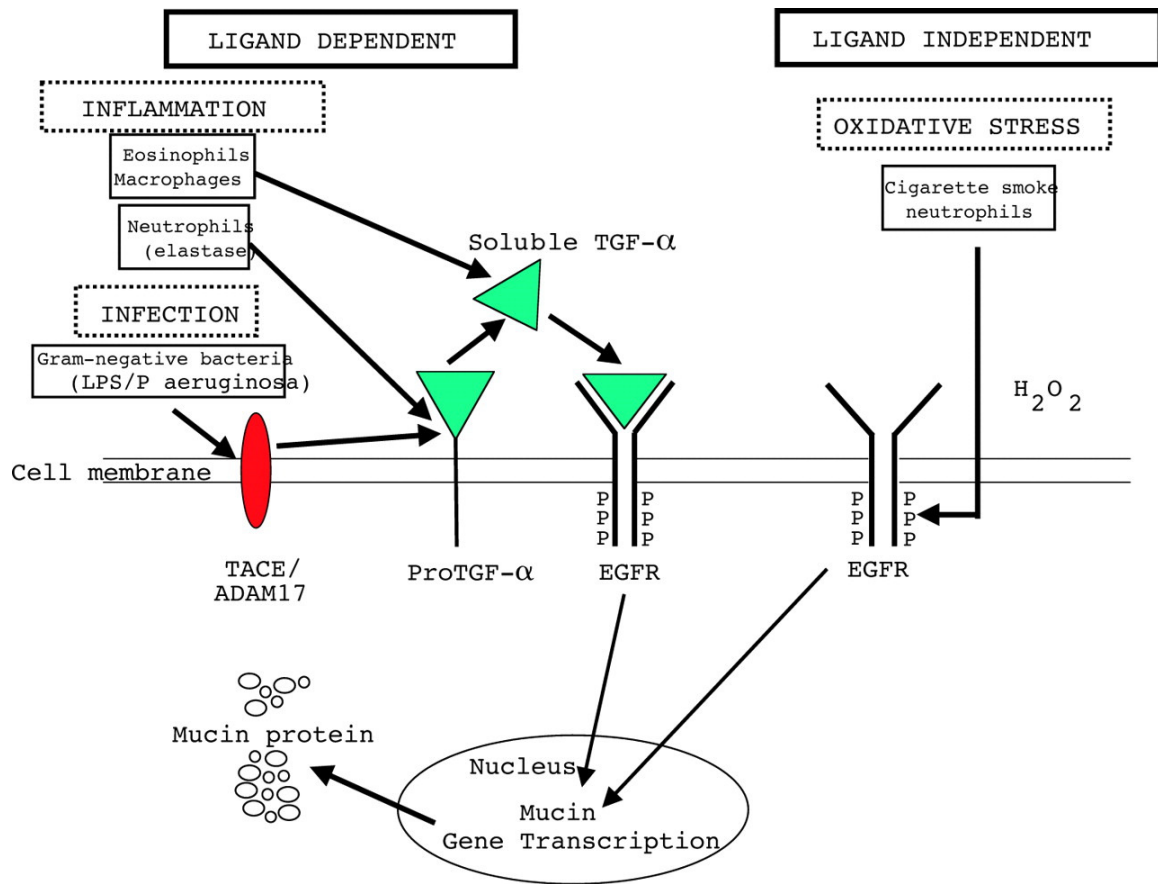


Figure 2.16: Ligand dependent and ligand independent EGFR activation leading to mucin synthesis (Burgel & Nadel, 2004).

2.6.7 Airway remodelling and lung function consequences

Small airways have been reported as the major site of obstruction in COPD in various studies (James C. Hogg et al., 2004; J. C. Hogg et al., 1968). Airway remodelling and chronic inflammation in these airways has been associated with airflow limitation (Bosken et al., 1990; M. Cosio et al., 1978; James C. Hogg et al., 2004). In addition, a strong association has been observed between disease progression and increased structural abnormalities in components of the airways including epithelium, lamina propria, airway smooth muscle and adventitia in COPD (James C. Hogg et al., 2004). Furthermore, chronic inflammation in peripheral airways has been associated with abnormal connective tissue deposition in the airway wall and with emphysema (M. Cosio et al., 1978). With increasing abnormalities in

the peripheral airways, lung function declined progressively and small airway function tests helped to differentiate patients with minimal but progressive pathologic and physiological changes from patients with normal airways (M. Cosio et al., 1978).

Emphysema in COPD may also play an important role in airflow limitation, depending on the type of the emphysema involved (Kian Fan Chung, 2005). Centrilobular or centriacinar emphysema results in destruction and dilation of respiratory bronchioles in the central portions of the acinus, is usually related to tobacco smoke and is primarily severe in the upper lobes of the lung and associated with small airway obstruction. Panlobular or panacinar emphysema on the other hand involves a more uniform destruction and dilation over the entire acinus and is usually associated with the lower lobes of the lungs (for example in $\alpha 1$ anti-trypsin deficiency) (J. C. Hogg, 2004; W. D. Kim et al., 1991; M. Saetta, 2006) and its major impact is on lung diffusing capacity.

Panacinar emphysema is also related to loss of lung elasticity and higher compliance, whereas centrilobular emphysema is associated with chronic airway inflammation and airway hyper-responsiveness (Kian Fan Chung, 2005). Hale K. A. et al reported that severe pathological changes, including emphysema, correlated with the degree of airflow limitation as measured by FEV₁ (Hale, Ewing, Gosnell, & Niewoehner, 1984). However in emphysema the loss of elastic recoil is the cause of airflow limitation rather than changes in airway wall remodelling directly on the airway lumen (J. C. Hogg et al., 1968). Where diffusing capacity is reduced in addition to airflow limitation, symptoms are appreciably greater (Burgess et al., 2008).

Recent detailed data on airway remodelling in COPD is very sparse, and how these changes lead to decline in lung function are not well understood. It is likely that in COPD the epithelium may get damaged by cigarette smoke and airway wall remodelling may occur in response to this, but the mechanisms and detailed structural changes are poorly described. In other COPD-like airway disease, especially bronchiolitis obliterans syndrome (BOS, a manifestation of chronic rejection post lung transplant), a new component of remodelling is emerging in which alveolar epithelial cells undergo a transition to a mesenchymal phenotype with

myofibroblast characteristics and then migrate through the Rbm to the sub-epithelial lamina propria, a process termed “epithelial-mesenchymal transition” (EMT) (Borthwick et al., 2009; Ward et al., 2005)}. There are no reported studies on EMT in COPD, but a significant body of literature shows evidence of EMT in BOS (Borthwick et al., 2009; Ward et al., 2005) and also in idiopathic pulmonary fibrosis (IPF) (Brigham C. Willis et al., 2006). Hackett et al, recently reported that alveolar epithelial cells from asthmatics can undergo a transition to a mesenchymal phenotype when stimulated with TGF- β 1, but when the actual airway biopsy sections from asthmatics were stained for markers of EMT they were negative and in addition the thickened homogenous Rbm in asthma shows none of the characteristic fragmentation, characteristic of EMT (T. L. Hackett et al., 2009).

2.7 Epithelial - mesenchymal transition (EMT)

From a general perspective, epithelial mesenchymal transition is a biological process in which epithelial cells undergo extensive molecular reprogramming allowing epithelial cells to undergo numerous biochemical changes and acquire a mesenchymal phenotype. This is accompanied by progressive loss of epithelial markers, gain in migratory potential and invasiveness, and enhanced capacity to produce extracellular matrix components (Figure 2.17) (Raghu Kalluri & Neilson, 2003; R. Kalluri & Weinberg, 2009). The digestion of underlying basement membrane facilitates the process of EMT and the fragmented anatomical changes in the Rbm have been reported as a marker of completion of the process of EMT (R. Kalluri & Weinberg, 2009). This, of course is what first attracted my attention to EMT being a possibility in COPD.

Elizabeth Hay from Harvard University was the first to describe the process of “epithelial mesenchymal transformation” in 1982 and then in 1995 as an important process involved in embryogenesis and organ development (Guarino, Tosoni, & Nebuloni, 2009; Hay, 1982, 1995). In the intervening time, the term “transformation” has been replaced with the term “transition”, indicating potential induction and reversibility of the process, even a two-way process (Raghu Kalluri & Neilson, 2003). The reverse process has been termed as mesenchymal-epithelial transition

(MET), but there are very few studies reporting MET in the literature and most are concerned with embryogenic kidney formation (R. Kalluri & Weinberg, 2009).

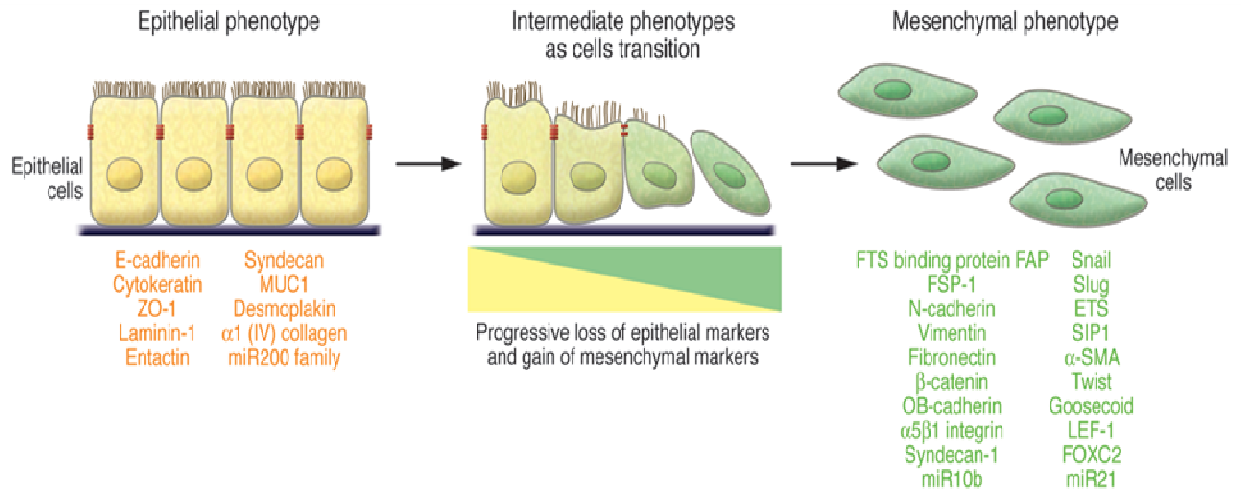
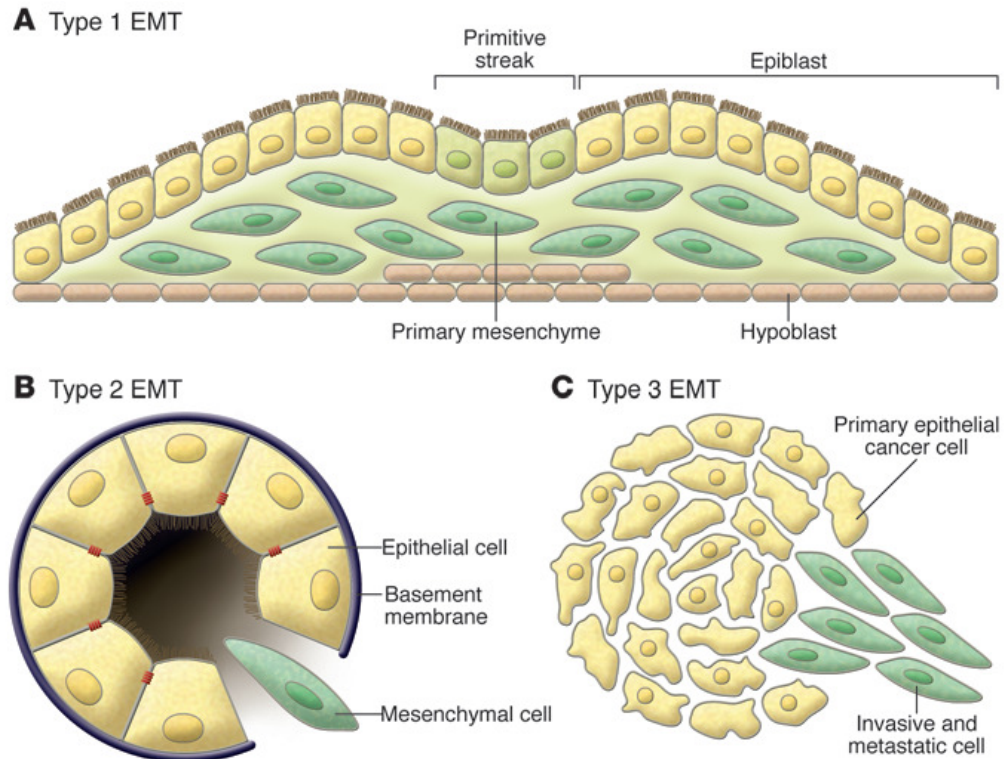


Figure 2.17: Epithelial mesenchymal transition, with progressive loss of epithelial markers and gain of mesenchymal markers (R. Kalluri & Weinberg, 2009).

The literature on these different terms is somewhat conflicting; they are often used inappropriately. Thus, the terms “epithelial mesenchymal transformation, interaction and transition are often confused and inappropriately used with the term epithelial mesenchymal transdifferentiation” (Raghu Kalluri & Neilson, 2003). “Transformation” is typically associated with the oncogenic conversion of the epithelium and is not usually plastic over time, whereas “transdifferentiation” is associated with differentiated cells changing into other differentiated cells. Also, epithelial mesenchymal “interaction” involves paracrine cross-talk between epithelial cells and stromal fibroblasts and is totally separate from the concept of epithelial mesenchymal “transition” (Raghu Kalluri & Neilson, 2003). Epithelial mesenchymal transition is considered as a type of transdifferentiation, but the term epithelial mesenchymal transition is preferred over transdifferentiation as the latter is associated with differentiated cells changing into other differentiated cells. This issue is still a matter of debate with no clear explanation available (Raghu Kalluri &

Neilson, 2003). For my work in COPD, I have maintained use of the term epithelial mesenchymal “transition” or EMT.

Recently, Raghu Kalluri et al (R. Kalluri, 2009; R. Kalluri & Weinberg, 2009; Zeisberg & Neilson, 2009) also from Harvard, suggested that EMT could be divided into three different types (Figure 2.18) based on the biological conditions in which



they occur and associated consequences:

Figure 2.18: Different types of EMT. (A) Type 1 EMT (B) Type 2 EMT (C) and Type 3 EMT, (R. Kalluri & Weinberg, 2009).

- *Type 1 EMT* occurs during implantation, embryogenesis and organ development, this type of EMT is not associated with organ fibrosis and metastasis, and however type 1 EMT has the potential for MET to form secondary epithelia.

- *Type 2 EMT* is implicated in wound healing, organ fibrosis and tissue regeneration. It occurs in response to injury and contributes to the repair process by generating fibroblasts and other cells to repair the tissue. This type of EMT is usually associated with inflammation and may stop once the inflammation ceases, but if the inflammation is long lasting due to repeated injury, type 2 EMT could well lead to organ damage and may have very severe consequences. Type 2 EMT could potentially be picked up using very reliable mesenchymal markers available like fibroblast-specific protein (also called S100A4), collagen I, along with vimentin, desmin, E-cadherins and cytokeratins.
- *The last type of EMT, type 3* is associated with cancer progression and metastasis. Cells produced by this type of EMT may invade through and into the circulation and produce metastases. A significant body of literature explains the signalling pathways involved with type 1 and type 2 EMT, but we don't know much about type 3 signalling pathways which are mainly involved with cancer progression.

2.7.1 Mechanism of EMT

EMT can be considered as a marker of profound epithelial plasticity engaged by disaggregating and reshaping epithelium for movement (B. C. Willis & Borok, 2007). The epithelium is basically a sheet of cells, in the airways one cell deep, with epithelial cells joined to each other in a uniform way. Tight junctions and adheren junctions between epithelial cells hold the epithelial cells strongly together as a single layer and restrict the movement of individual cells away from the epithelium. The epithelial cells are polarized, from which we understand that in epithelium apical and basal surfaces are likely to be visually different and may carry different functions. Mesenchymal cells compared to epithelial cells are lacking uniform composition and strong adhesion properties; they are of a more elongated and irregular shape allowing the cells to gain migratory potential for movement (J. M. Lee et al., 2006).

During transition the epithelium loses cell polarity, tight junctions like zonula occludens, adheren junctions like E-cadherins and desmosomes, cytokeratin filaments

(Cytokeratins are keratin containing intermediate filaments found in epithelial cells and are vital for regular tissue function and structure) and reorganize their actin fibres, emission of filopodia and lamellopodia and then finally go through a transition into an elongated and irregular morphology associated with gain of mesenchymal markers (e.g.: S100A4 and vimentin) simultaneously (Savagner, 2001; B. C. Willis & Borok, 2007).

The process of EMT can simply be induced by a combination of different cytokines and growth factors associated with dismantling of the basement membrane by proteolytic activity (Raghu Kalluri & Neilson, 2003). The cells undergoing transition have the capacity to secrete proteolytic enzymes from the metalloproteinase family, which can start the proteolytic digestion of the underlying basement membrane assisting migration of the cells (J. Yang & Liu, 2001). A diverse variety of growth factors are involved in the process, mainly TGF- β , EGF, insulin growth factor-II (IGF-II), hepatocyte growth factor (HGF) and also fibroblast growth factor-II (FGF-II) (Figure 2.19); they have the capacity to induce EMT by binding to different receptors on epithelium cells (Raghu Kalluri & Neilson, 2003).

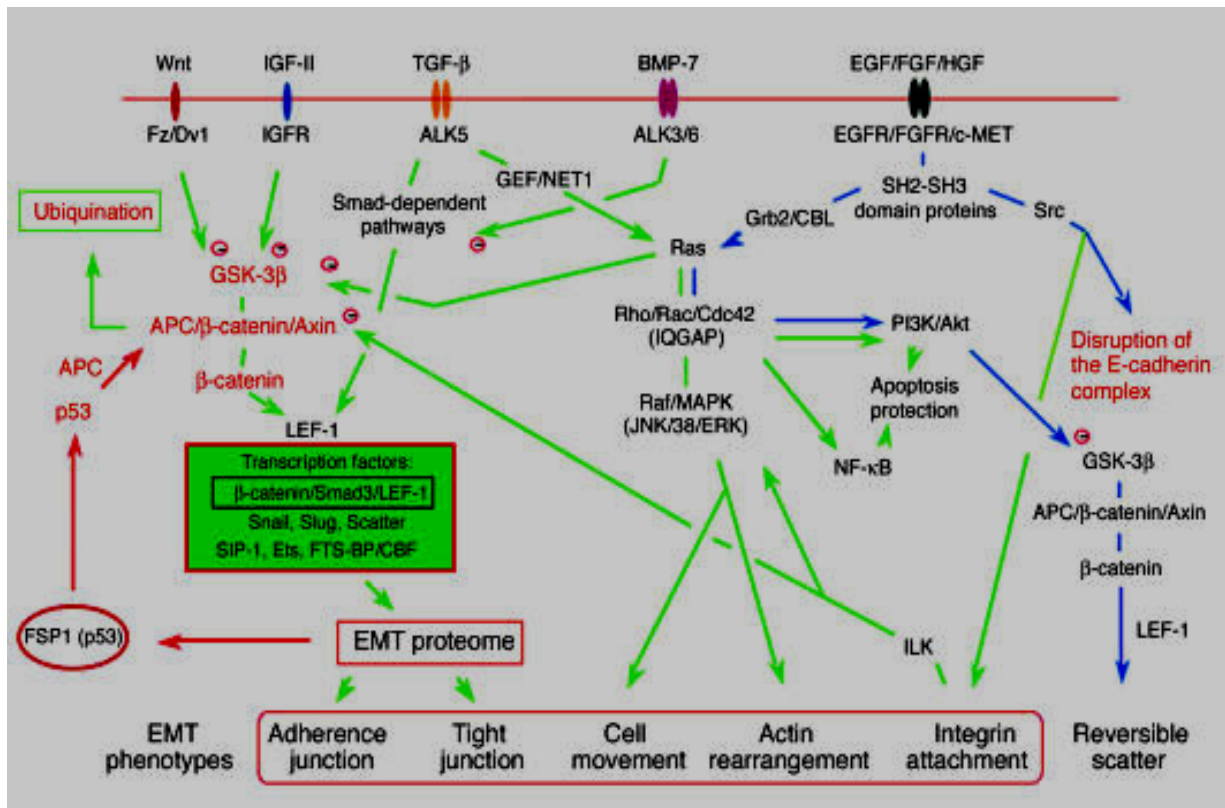


Figure 2.19: Schematic representation of different signalling pathways involved in EMT (Raghu Kalluri & Neilson, 2003).

TGF- β has been considered a prototypical driver of EMT; Fan et al (Fan et al., 1999) reported that TGF- β can induce the differentiation of tubular epithelial cells into α -SMA positive myofibroblasts. It was also reported that TGF- β and FGF-II expression is central to MMP-2 and MMP-9 expression; these proteolytic enzymes have the capacity to digest the basement membrane/Rbm to facilitate the migration of cells (Raghu Kalluri & Neilson, 2003; Ward et al., 2005). TGF- β can act mainly in two ways, through Smad-3 dependent pathway or other Smad independent pathway (B. C. Willis & Borok, 2007). Depending on the type of tissue all three isoforms of TGF- β could be involved during transition (Raghu Kalluri & Neilson, 2003). Other studies have also shown that TGF- β expression is up-regulated in lungs of patients with idiopathic pulmonary fibrosis (IPF) and can enhance EMT (B. C. Willis & Borok, 2007). On the other hand, a study reported by Zeisberg et al revealed that bone morphogenic protein (BMP)-7 which is a member of the TGF- β superfamily has the ability to reverse TGF- β -induced EMT by restoring E-cadherin levels to normal through the Smad pathway, mainly through Smad 5, but Smad 1 and Smad 8 can also transduce BMP-7 actions (Zeisberg et al., 2003).

Citterio et al reported that EGFR expression increases in the epithelium during the process of EMT and interaction between TGF- β and EGFR might play a key role in EMT by assisting in migration and matrix turnover (Citterio & Gaillard, 1994). Furthermore, Lo et al reported that EGF/EGFR signalling pathways can lead to EMT via STAT3-mediated (signal transducer and activator of transcription 3) up-regulation of TWIST gene expression (Lo et al., 2007). IGF-II signalling pathways relocate the β -catenins (which belong to the family of cell adhesion molecules and is a subunit of the cadherin complex) from cells surface leading to the nuclear internalization resulting in intracellular degradation of the E-cadherin complex which further facilitates migration of cells as the epithelium becomes loose (Morali et al., 2001).

Janda et al (Janda et al., 2002) made an effort to differentiate true EMT from an epithelial phenocopy termed “reversible scatter”. Reversible scatter is similar to EMT but not true EMT, but more like a brief episode of EMT. For example Hackett et al, (T. L. Hackett et al., 2009), recently reported that alveolar epithelial cells from asthmatics can undergo a transition to a mesenchymal phenotype when stimulated

with TGF- β 1, but when the actual airway biopsy sections from asthmatics were stained for markers of EMT they were negative. Thus, this is not true EMT, but probably reversible scatter as a result of TGF- β 1 stimulation for a short period of time, and occurs only temporarily following cytokine stimulation with cells assuming spindle like cell morphology, loss of epithelial markers and gain of mesenchymal markers for a brief period of time. However, as the stimulus is removed the epithelium returns to its original shape and the whole process ceases. They also reported that true EMT is mainly induced by TGF- β and Ras signalling pathways, whereas EGF, HGF and FGF favour scattering only, and are not able to produce classical EMT.

2.8 Signalling pathways in EMT

The epithelial signalling which drives the process of EMT is very complicated (Figure 2.19). Further, these signalling pathways are linked to each other with broad cellular consequences (Raghu Kalluri & Neilson, 2003). Brigitte Boyer et al suggested that when EMT is not activated by an oncogenic stimulus, then it is primarily induced by specific growth factors or extracellular matrix components binding to their associated cellular receptors leading to a strategic intrinsic kinase activation which sets the process off (Boyer, Valles, & Edme, 2000). The major signalling pathways involved in EMT are: TGF- β signalling pathways, the Wnt/ β -catenin signalling pathway, the Ras pathway and the Notch signalling pathway. All these pathways are extensively inter-related (Boyer et al., 2000; Guarino et al., 2009; Raghu Kalluri & Neilson, 2003).

2.8.1 TGF- β signalling pathway

I have already introduced transforming growth factor beta (TGF- β). It is a multifunctional protein playing a range of different roles through regulating tissue morphogenesis and differentiation by modulating cell proliferation, differentiation, cell adhesion, apoptosis, migration of cells, vasculogenesis and angiogenesis and is also involved in extracellular matrix turnover (Dennler, Goumans, & ten Dijke, 2002). TGF- β is a member of a large superfamily of structurally related proteins consisting of more than 30 members in mammals, including three types of TGF- β ,

four activins and over more than twenty bone morphogenetic proteins (BMPs) (Dennler et al., 2002). TGF- β has been implicated as a master switch in the induction of fibrosis in various organs including the lungs (B. C. Willis & Borok, 2007). Signal transduction associated with TGF- β involves two different pathways in EMT, Smad-dependent signaling pathway and smad-independent signaling pathway (Dennler et al., 2002; B. C. Willis & Borok, 2007).

Smad-dependent pathway: Most members of the TGF- β superfamily signal through cell-surface receptors of the serine/threonine kinase category. A family of proteins known as Smads are involved in transducing the ligand-induced signals from the cell surface to the nucleus of the cell (Flanders, 2004). Thus, Smads are mainly signalling proteins that have the capacity to modulate the activity of TGF- β ligands. The name Smads was derived from a contraction of the names of TGF- β like ligand signalling intermediates first recognized in *Drosophila* (Mad) and *Caenorhabditis elegans* (Sma) (Flanders, 2004). Eight different types of Smads have been reported, further grouped into three subfamilies. The first five include receptor-regulated Smads (Smad 1, Smad 2, Smad 3, Smad 5 and Smad 8/9), termed as R-Smads; one common mediator Smad (Smad 4), named Co-Smads; and two inhibitory Smads (Smad 6 and Smad7), termed I-Smads (Flanders, 2004; Wikipedia).

The Smad-dependent pathways in the process of EMT in response to TGF- β are mainly mediated by Smad 3. In this pathway, TGF- β -generated signals are mediated by type I and type II serine-threonine kinase receptors and this process is initiated by binding of extracellular TGF- β ligands to the type II receptor on the cell surface. This binding further leads to the recruitment of type I receptors to form a heteromeric complex; at this stage the type I receptor is phosphorylated by the type II receptor, resulting in its activation. The type I receptor later phosphorylates Smad 2 and Smad 3 at serine residues inducing association with Smad 4 (Co-Smad) and forming a heteromeric complex leading to the translocation of this complex to the nucleus. In the nucleus this complex can act as a transcription factor and can also interact with other transcription factors leading to the activation or regulation of TGF- β responsive genes, which may include, Snail, connective tissue growth factor (CTGF), α -SMA (α -smooth muscle actin), collagen 1A2 and also plasminogen activator inhibitor-1

(PAI-1). Smad proteins also have the potential to combine with the lymphoid enhancer factor-1 (LEF-1) which is also an important transcription factor involved in EMT. LEF has binding sites for both Smads and β -catenin, formation of this complex there after leads to the activation of various other transcription factors which further lead to transcription of genes associated with the EMT proteome. TGF- β -activated Smad proteins can also activate the integrin-linked kinase (ILK) or integrin signalling leading to the suppression of E-cadherin by expression of the β -catenin/LEF complex, and this process may enhance the expression of fibronectin and proteolytic enzymes from the matrix metalloproteinase family (Guarino et al., 2009; Raghu Kalluri & Neilson, 2003; Postma & Timens, 2006; B. C. Willis & Borok, 2007).

Smad-independent pathway: The Smad-independent pathway is not as well studied compared with the Smad dependent pathway in EMT although a significant body of *in-vitro* evidence is available for the role of a Smad independent pathway in EMT. It is hard to differentiate these pathways clearly as there is potential for significant cross-talk between Smad proteins and non-Smad proteins during EMT. The Smad-independent pathway mainly includes RhoA, Ras MAPK (mitogen-activated protein (MAP) kinases), PI3 kinase (Phosphatidylinositol 3-kinases), Notch and Wnt/ β -catenin signalling pathways. Although the Smad-dependent pathway is considered as the main pathway, depending on the type of tissue it may not be sufficient to induce full blown EMT without activation of other supplementary pathways (Zavadil & Bottinger, 2005). Major Smad-independent pathways are discussed separately below.

2.8.2 The Wnt/ β -catenin signalling pathway

The Wnt signalling pathway is activated when Wnt proteins bind to specific cell surface receptors. “The name *Wnt* was coined as a combination of *Wg* (wingless gene, originally identified in *Drosophila melanogaster*) and *Int* (Int gene was originally identified as vertebrate genes near several integration sites of mouse mammary tumor virus)” (Wikipedia). The Wnt signalling pathway describes a network of Wnt proteins, which form a family of highly conserved secreted signaling molecules that regulate cell-to-cell interactions during embryogenesis and cancer. Wnt-mediated signalling (Figure 2.19) can inhibit phosphorylation of β -catenin by

glycogen synthase kinase 3 β (GSK-3 β), hence stabilizing cytoplasmic levels of β -catenin, this leads to the translocation of β -catenin to the E-cadherin complex or into the nucleus where it combines with the lymphoid enhancer factor-1 (LEF-1) and other transcription factors leading to the activation of a diverse variety of EMT-associated genes, including those encoding for fibroblast specific protein-1 (FSP-1 or S100A4), vimentin, fibronectin, MMPs and also members from the Snail family. On the other hand, in the absence of Wnt-associated signals most of the β -catenin is bound to the E-cadherin complex, while the unbound β -catenin undergoes phosphorylation by GSK-3 β which further allows it to form a complex with APC suppressor protein and Axin (Adenomatosis polyposis coli or APC is a protein encoded by the APC gene in humans; it is suggested that APC protein plays an important role in cell migration by targeting β -catenin for proteasomal degradation) (Nathke, 1999).

p53 activates an APC-dependent pathway and both of these lead to the direct loss of β -catenin through proteasomal degradation. In this whole process free levels of β -catenin are regulated by E-cadherin complexes or by APC/ β -catenin/Axin complexes, the latter shuttle β -catenin between its degradation through proteasomal pathways and adheren junctions in EMT. β -catenin plays a dual role in the EMT process: it has the potential for cell-cell adhesion when bound to the cadherin complex, and on the other hand can also act as a transcription factor when present in the nucleus. The cell adhesion due to β -catenin depends on binding of β -catenin to the α -catenin and further binding of the α -catenin to the cadherin. Furthermore, in addition to Wnt glycoproteins, IGF-II, Ras and integrin-linked kinase (ILK) all lead to β -catenin accumulation in the cytoplasm and also increased Snail, possibly by GSK-3 β or by inhibition of other kinases to facilitate EMT (Guarino et al., 2009; Raghu Kalluri & Neilson, 2003; Nathke, 1999; Postma & Timens, 2006; B. C. Willis & Borok, 2007; Zavadil & Bottinger, 2005).

2.8.3 The Ras signalling pathway

Ras is a superfamily of small GTPases or G-proteins. All the signalling pathways involved with EMT can activate small GTPases through ligand-inducible receptor

kinase activation. These small GTPases are involved with changes in cell shape and gain of migratory potential. Transition of epithelium to mesenchymal phenotype depends considerably on different molecular switches under the control of small GTPases from the Ras superfamily. Small GTPases are activated by guanine nucleotide exchange factors and deactivated by GTPase activating proteins. There are more than a hundred proteins in the Ras superfamily but the 3 best studied small GTPases are, Rho, Rac and Cdc42, and they can activate or inhibit each other during the process of EMT.

Rho signal transduction pathways are linked to the actin cytoskeleton and are also helpful in rearranging the actin stress fibres and can stimulate actin-myosin contraction in the cell body. Furthermore, they also participate in regulation of cell polarity, gene transcription, cell cycle, vesicular transport pathways and are also involved with various enzymatic activities. Rac on the other hand has the capability to induce actin-rich surface extensions called lamellopodia. Cdc42 supports the development of actin-rich finger-like protrusions called filopodia and modulates cellular asymmetry. The small GTPase family is able to control or regulate different cellular properties including contraction, migration, proliferation, and phagocytosis. These cellular activities of small GTPases can potentially activate MAP kinases, altering gene transcription and are vital to change in cell phenotype during the process of EMT. (Bar-Sagi & Hall, 2000; Etienne-Manneville & Hall, 2002; Guarino et al., 2009; Raghu Kalluri & Neilson, 2003; Savagner, 2001).

2.8.4 The Notch signalling pathway

The Notch pathway plays an important role in EMT and functional interactions between Notch signalling and EGF, FGF, TGF- β and Smads have been suggested (Dasari, Gallup, Lemjabbar, Maltseva, & McNamara, 2006; Zavadil, Cermak, Soto-Nieves, & Bottinger, 2004). It is a highly preserved cell signalling system present in most multicellular organisms and is a family of four different types of receptors, Notch1-4. Different ligand proteins can bind to the extracellular domain of the Notch receptor and can induce proteolytic cleavage and release of the intracellular domain, which then enters the nucleus of the cell to alter the gene expression. The first mutant of Notch was identified in *Drosophila melanogaster* (Artavanis-Tsakonas, Rand, & Lake, 1999). Zavadil et al demonstrated that when keratinocytes were induced to

EMT by TGF- β , Notch signalling pathway activation occurred downstream of TGF- β , controlled by early up-regulation of the Notch ligand, Jagged1 and also the Notch target genes TLE3 (transducin-like enhancer) and HES1 (hairy enhancer of split) (Zavadil et al., 2001).

In 2004, the same group described the expression of the HES1-related (Dasari et al., 2006) transcriptional repressor family which included HEY1, HEY2, HES1 and HES5 and the Notch ligand jagged1 being induced by TGF- β at the start of EMT in a panel of epithelial cells from kidney tubules, mammary glands and epidermis (Zavadil et al., 2004). They also reported that TGF- β -induced EMT could be inhibited by suppressing the expression of the Notch ligand Jagged1 and HEY1 and also by chemical inactivation of Notch (Zavadil et al., 2004).

2.9 Transcriptional control of EMT

Signalling pathways implicated in the process of EMT end up as a final common path in the nucleus of the cell where they regulate the transcription of EMT-related genes. In EMT a wide array of different proteins are gained, maintained or attenuated, which requires the coordinated expression of numerous set of genes. Therefore, it becomes quite critical to understand and identify the different molecular targets of these signalling pathways leading to EMT (Boyer et al., 2000). A range of different transcriptional factors are involved in regulating EMT; among these several transcription factors can act as master switches in regulating the whole process of EMT (Zavadil & Bottinger, 2005), with a degree of synergy and/or redundancy between them.

2.9.1 The Snail family

The *Snail family* of transcription factors has been implicated in EMT; it includes Snail or Snail1 and Slug or Snail2 zinc finger proteins, and recently Snail3 has also been found. The Snail family induces EMT mainly by repressing transcription by recognizing E-box elements (they are “enhancer” elements and play a regulatory role in control of gene transcription) in their associated target promoters. It is reported that Snail can repress transcription of the E-cadherin gene through several E-boxes located in the E-cadherin promoter in cultured cells and also during embryonic

development and tumour progression (Barrallo-Gimeno & Nieto, 2005; Xu, Lamouille, & Derynck, 2009; Zavadil & Bottinger, 2005). Snails themselves are regulated by a variety of signalling pathways and at numerous levels. For example, it is reported that selective phosphorylation by GSK3 leads to the inhibition of Snails by ubiquitination or degradation. On the other hand Wnt signalling pathway can inhibit GSK3 resulting in Snail activation and loss of E-cadherins.

Various growth factors or signalling molecules involved in EMT can also regulate Snails. For example, TGF- β has the potential to activate both Snail and Slug directly through a Smad3-dependent pathway (Zavadil & Bottinger, 2005). TGF- β 1 can induce Snail1 expression in mesothelial cells, epithelial cells and hepatocytes and also during palate development (Xu et al., 2009). TGF- β 2 and BMP-4, on the other hand, can induce expression of Snail2 or Slug (Zavadil & Bottinger, 2005). EGF has also been reported in several studies as an inducer of Snail and EMT. The EGF pathway promotes caveolin-dependent endocytosis which leads to loss in E-cadherin function and successive Snail and EMT activation (Lu, Ghosh, Wang, & Hunter, 2003). In addition, EGF can also activate STAT3 ("signal transducer and activator of transcription 3", it is a transcription factor encoded by STAT3 gene in humans), which has the potential to enhance Snail function (Barrallo-Gimeno & Nieto, 2005).

Another Zinc-finger protein from the Snail family widely reported in EMT is Slug or Snail2, which can play an important role in regulating EMT (Savagner, 2001). Slug can be quickly induced in epithelial cells undergoing EMT by FGF and HGF and effectively regulates disassembly of desmosomes (Savagner, 2001; Zavadil & Bottinger, 2005). During EMT it is suggested that Slug appears to be activated through Ras/MAPK pathway and does not seem to be participating in E-cadherin suppression but rather contributes more to the maintenance of the mesenchymal phenotype (Boyer et al., 2000; Savagner, 2001). But interestingly it is also suggested that dissociation of E-cadherin junctions can lead to autoregulatory induction of Slug with successive suppression of E-cadherin transcription, although its exact role is still a matter of debate (Zavadil & Bottinger, 2005).

2.9.2 Role of Transcriptional repressors (DeltaEF1, SIP1 and Fos)

Delta-crystalline enhancer factor-binding factor 1 (DeltaEF1) (also known as ZFH1 and ZEB1), and Smad-interacting protein-1 (SIP1) (also known as ZFH2) are two closely related zinc finger *transcriptional repressors*. DeltaEF1 is a very close homologue of SIP1 and both have the capability to regulate E-cadherin expression during carcinogenesis associated EMT (Type 3 EMT) (Eger et al., 2005; Zavadil & Bottinger, 2005). SIP1 expression can be induced in response to TGF- β and has the potential to regulate the activity of Smad proteins. Comijn et al have shown that ectopic expression of SIP1 in MDCK cells (Madin-Darby canine kidney cell line) leads to the destruction of adheren junctions, especially E-cadherin, favouring invasion in malignant epithelial tumours (Comijn et al., 2001; Zavadil & Bottinger, 2005).

Eger and colleagues (Eger et al., 2005) strongly suggested that DeltaEF1 has the potential to directly inhibit E-cadherin transcription, being associated with the E-cadherin promoter at the chromatin level. They also suggested that although E-cadherin suppression is considered as a hallmark of EMT, it is still not itself sufficient to induce the expression of N-cadherin and vimentin, EMT-related mesenchymal markers. Thus DeltaEF1 may play an important role in regulating epithelial plasticity by controlling other proteins, but cannot induce EMT fully on its own.

Reichmann and colleagues showed that Fos transcription factor expression in mammary epithelial cells can lead to the process of EMT, by modulating epithelial phenotype and increasing invasiveness (Reichmann et al., 1992). Zavadil and colleagues reported that in human keratinocytes, TGF- β stimulates the ERK-dependent induction of c-Fos, which occurs very rapidly but for only a short period of time at the inception of sustained EMT (Zavadil et al., 2001), when it may have a very strategic role.

2.9.3 Id, Twist and E2A

Id proteins (Id1, Id2, Id3, and Id4) are recognized as inhibitors of differentiation, playing a vital role during carcinogenesis (Kowanetz, Valcourt, Bergstrom, Heldin, & Moustakas, 2004). Kowanetz et al did a microarray analysis of epithelial cells and

reported that TGF- β 1 suppresses Id2 and Id3 for a long period of time, whereas BMP7 induced these factors. In addition, Id2 also inhibited the destruction of adherens and tight junctions in TGF- β -induced EMT (Zavadil & Bottinger, 2005). This suggests that TGF- β may favour EMT by suppressing these inhibitors of differentiation and BMP-7, but still the exact physiological role of these pathways is poorly understood (Kowanetz et al., 2004; Zavadil & Bottinger, 2005). Id proteins associate constitutively with the basic helix-loop-helix (bHLH) transcriptional regulator E2A, and maintain epithelial phenotype by inhibiting the role of E2A in E-cadherin suppression. Conversely, TGF- β -mediated inhibition of Id proteins can lead to activation of E2A (a transcription factor), maintaining the mesenchymal phenotype during EMT (Zavadil & Bottinger, 2005).

Another basic helix-loop-helix transcription factor that appears to be centrally associated with EMT is Twist, which is an important trigger factor involved in embryogenesis and carcinogenesis (Pozharskaya et al., 2009; Zavadil & Bottinger, 2005). Twist can be induced during EMT by the expression of EGFR, TGF- β and the transcription factor nuclear factor κ B (NF- κ B). Pozharskaya et al reported in a recent study that Twist contributes to EMT in the model of virus-induced lung fibrosis (Pozharskaya et al., 2009). Yang and colleagues reported that Twist suppression in mammary carcinoma cells specifically inhibits their ability to metastasize from the mammary gland to the lung; they also reported that ectopic expression of Twist in MDCK cells leads to loss of E-cadherin with subsequent expression of mesenchymal markers (e.g. vimentin, α -SMA and N-cadherins) and an increase in migratory potential, thus playing a very important role in tumour metastasis (type-3 EMT) (J. Yang et al., 2004; Zavadil & Bottinger, 2005). In another study, Kida et al reported that Twist was associated with renal tubular EMT leading to proliferation of myofibroblasts and subsequent renal fibrosis in an obstructed kidney model (Kida, Asahina, Teraoka, Gitelman, & Sato, 2007), but there are very few studies reporting role in EMT associated with chronic inflammation (Mironchik et al., 2005).

2.10 The EMT proteome

The EMT proteome is defined as the essential changes in proteins gained, maintained or attenuated during the EMT process (Figure 2.20). This involves a wide range of

proteins and most of the studies reported in the literature have been able to focus on a few specific markers and are far from complete. The majority of studies suggest that transcriptional factors, especially from the Snail family are the main players in the events leading to the loss of epithelial markers (e.g. E-cadherin, muc-1, cytokeratin and desmosomes) and gain of mesenchymal markers (e.g. FSP-1, or S100A4, vimentin, fibronectin and N-cadherin) with increased mobility (Raghu Kalluri & Neilson, 2003), but actually very little attention has been given to clearly identifying EMT *in vivo* and *in vitro*, and the best/most specific markers to use are still a matter of debate. Michael Zeisberg and colleagues have made an attempt to define the *in vivo* and *in vitro* criteria to identify EMT in this way (Figure 2.21) (Zeisberg & Neilson, 2009).

EMT has been widely studied in different cell culture systems and most of the attention has been given to the process of identifying cells undergoing transition to a fibroblast like phenotype. One of the benefits associated with an *in vitro* system is the ability to detect the phenotype and movement of the cells in the culture medium, but on the other hand it is much more difficult in an *in vivo* system because many of the tissue sites are disrupted by inflammation and cicatrisation (Zeisberg & Neilson, 2009). Unfortunately, because something can be demonstrated in a manipulated *in vitro* system and environment, does not necessarily mean that those change(s) are operative *in vivo*.

The EMT proteome

Proteins gained or maintained:

Snail
Slug
Scratch
SIP1
E47
Ets
FTS binding protein
RhoB
FSP1
TGF- β
FGF-1,-2,-8
MMP-2
MMP-9
Vimentin
 α SMA
Fibronectin
Collagen type I
Collagen type III
Thrombospondin
PAI-1

Proteins attenuated:

E-cadherin
 β -catenin
Desmoplakin
Muc-1
ZO-1
Syndecan-1
Cytokeratin-18

Figure 2.20: The EMT proteome, showing proteins gained or maintained and lost during EMT (Raghu Kalluri & Neilson, 2003).

In vivo criteria for EMT

Major criteria

- Use of an epithelial cell reporter construct that appears locally in newly formed fibroblasts
- New expression of FSP1 and possibly DDR2 associated with disruption of basement membrane
- Increased expression of HSP47, collagen I ($\alpha 1$), collagen 2 ($\alpha 2$), N-cadherin, or vimentin
- Nuclear relocalization of CBF-A or β -catenin/LEF or new expression by in situ hybridization of one of the following transcription factors: Snail, Slug, or Twist
- Loss or partial loss of epithelial markers such as cytokeratin, E-cadherin, or ZO-1
- Spindle-shape morphology with redistribution of stress fibers and loss of polarity

Minor criteria

- Localized adjacency of transitioning cell near its epithelial compartment
- Exclusion of possible bone marrow-derived progenitor cells

In vitro criteria for EMT

Major criteria

- New expression of FSP1 and possibly DDR2
- Increased expression of HSP47, collagen I ($\alpha 1$), collagen 2 ($\alpha 2$), or vimentin
- Cadherin switch
- Nuclear relocalization of CBF-A or β -catenin/LEF or new expression of one of the following transcription factors: Snail, Slug, or Twist
- Absence of epithelial markers; loss of cytokeratin or ZO-1
- Spindle-shape morphology with redistribution of stress fibers and loss of polarity
- Resistance to apoptotic stimuli
- Increased migratory capacity
- Phenotype stable upon removal of inducing stimulus

Minor criteria

- Abundant intermediate filaments and microfilaments
- Loss of chromatin condensation associated with gain of multiple nucleoli
- Gain of rough ER, abundant lysosomal granules, and loss of intercellular junctions on electron microscopy

Figure 2.21: *in vivo* and *in vitro* criteria from EMT (Zeisberg & Neilson, 2009).

Thus, an ideal way to identify EMT associated with chronic inflammation is to use reporter genes in cultured epithelial cells that can be tracked to fibroblasts on transition. However, marking studies are very difficult to perform with human tissue, and in this setting the ideal approach is to identify epithelial cells positive for S100A4, a fibroblast specific protein-1 (also known as FSP-1) associated with partial or complete loss of cytokeratins or E-cadherins, lying adjacent to the typically disrupted and fragmented basement membrane or Rbm (Zeisberg & Neilson, 2009), evidence of the effects of the proteolytic activity of matrix metalloproteinases

(MMPs) (Ward et al., 2005) which are an inherit part of this process. This basement membrane or Rbm change has been suggested as the best “hallmark” of EMT *in vivo* (R. Kalluri & Weinberg, 2009). Alpha-smooth muscle actin has also been observed in mature fibroblasts, but is non-specific and is not present in newly transitioning epithelial cells, whereas S100A4 is mainly confined to transitioning epithelial cells and is a more reliable and specific marker than alpha-SMA (Zeisberg & Neilson, 2009). Elizabeth Hay et al (Hay, 2005) argued that mesenchymal cells and fibroblasts shouldn't be defined on the basis of alpha-SMA stress fibres, as myofibroblast phenotypes are not thought to migrate actively. Another marker of EMT widely reported is an intermediate filament protein called vimentin, which has the potential to induce changes in cell shape, motility, and adhesion during EMT (Mendez, Kojima, & Goldman). Vimentin has also been suggested to play a major role in breast cancer progression, an observation made both *in vitro* and *in vivo* (Kokkinos et al., 2007).

2.10.1 S100A4 (or FSP-1)

S100A4 belongs to a large family of Ca^{2+} -binding proteins collectively designated as S100. There are at least 21 described members in the S100 family which includes two newly discovered members, namely S100A14 and S100Z. Usually S100 proteins are low molecular weight proteins ranging between 9 to 13 kDa, sharing a common feature of two Ca^{2+} -binding EF-hand motives (helix-loop-helix), and most of the members exist as dimmers, including S100A4. S100 proteins do not have any enzymatic activity (Rosario, 2003; Schneider, Hansen, & Sheikh, 2008).

The most widely reported protein in EMT from the S100 family is S100A4, and it is considered as a potential mediator of EMT and organ fibrosis in diseases. S100A4 has both intracellular and extracellular effects leading to a wide range of biological actions including increasing contractility, motility, differentiation and cellular growth (Rosario, 2003; Schneider et al., 2008).

Raghu Kalluri et al suggested that S100A4 in the cytoplasm dimerizes and binds to the c-terminal of p53 (it is a tumour suppressor protein encoded by TP53 gene in humans), in the presence of calcium and this process may guard p53 from the APC-mediated destruction pathway and may raise levels of free β -catenin (Figure 2.19),

thus facilitating and maintaining the process of EMT. Therefore, the exact role of S100A4 is not clear but it also has the potential for angiogenesis (Raghu Kalluri & Neilson, 2003), and seems well fitted to be a core mediator of the changes associated with chronic inflammation as well as metastatic tumours. Indeed, it may be a link between the two processes in diseases such as COPD or IPF.

S100A4 has been suggested as a prototypical and very reliable cytoskeletal marker for detecting EMT in various pulmonary disorders and cancer (Lawson et al., 2005; Ward et al., 2005; Zeisberg & Neilson, 2009). Preliminary findings in idiopathic pulmonary fibrosis showed strong staining for S100A4 in fibroblasts (Schneider et al., 2008), Chris Ward et al showed that S100A4 localises to bronchial epithelial cells in human transplanted lungs indicative of EMT in the airways which may further lead to remodelling and fibrosis as part of chronic rejection (Ward et al., 2005). This was further confirmed by a study by Hodge and colleagues (Hodge et al., 2009).

There is emerging evidence that S100A4 can also activate expression and release of MMPs which in turn may lead to tissue remodelling and assisting in the migration of cells by degrading the basement membrane and Rbm and components of the extracellular matrix (Raghu Kalluri & Neilson, 2003; Schneider et al., 2008).

In a very interesting study (in prostate cancer), Saleem and colleagues observed that S100A4 gene repression significantly decreased the expression of MMP-9, while the over expression of the S100A4 gene considerably increased MMP-9 expression. These expression changes were mirrored in changes in proteolytic activity of MMP-9. They also suggested that transcriptional activation of MMP-9 is regulated by the S100A4 gene, as knock down of the S100A4 gene decreased MMP-9 activity, while over expression lead to an increase in MMP-9 promoter activity (Saleem et al., 2006). Increased expressions of S100A4 and MMP-9 are also observed in human non-small cell lung cancer and have significant correlations with clinical and biological behaviour of these cancer cells (Chen, Wang, Zhang, Chen, & Sun, 2008).

Put together all these data suggests that S100A4 could be involved both in inflammation-related fibrosis and in cancer progression. Tissue fibrosis and cancer share common signalling pathways, molecular and biological programs, and

especially EMT. This raises a possibility that S100A4 could be a common mediator of both fibrosis and cancer by directing EMT (Hodge et al., 2009; Okada, Danoff, Kalluri, & Neilson, 1997; Saleem et al., 2006; Schneider et al., 2008; Ward et al., 2005; Zeisberg & Neilson, 2009). “*Simply stated, S100A4 is central to EMT in diseases*” (Schneider et al., 2008).

2.10.2 Matrix metalloproteinases-9 (MMP-9)

MMP-9 or matrix metalloproteinases-9 (MMP-9) is a proteolytic enzyme from a family of more than twenty zinc dependent proteases, they all share some structural similarities but differ from each other in their expression profile and substrate specificity (Chakrabarti & Patel, 2005; Elkington & Friedland, 2006). Since MMPs have a broad range of substrate specificity they have the potential to cause significant damage to the host tissue, so their expression is tightly regulated at transcriptional level. MMPs are secreted as proenzymes which further require proteolytic cleavage to become active, but in the tissue they are tightly regulated by tissue inhibitors of metalloproteinases (TIMPs). TIMPs block the enzymatic activity of the MMPs and equilibrium between these two determines matrix turnover (Chakrabarti & Patel, 2005; Elkington & Friedland, 2006). Another physiological inhibitor reported for MMPs is α -2 macroglobulin (Lagente et al., 2005).

MMP-9 is also known as gelatinase B, as gelatin is one of its major enzymatic substrate; it also has the potential to degrade other components of the ECM such as type IV collagen, aggrecan, elastin and vitronectin. Human MMP-9 is highly glycosylated with a molecular mass of 92 kDa as a pro-enzyme and 83 kDa when active (Atkinson & Senior, 2003; Chakrabarti & Patel, 2005). MMP-9 is secreted by a variety of cells in the lungs including bronchial epithelial cells, macrophages, eosinophils, mast cells, natural-killer cells, dendritic cells, neutrophils, fibroblasts, smooth muscle cells, alveolar type II cells and endothelial cells (Atkinson & Senior, 2003).

Increased MMP-9 expression has been reported in various respiratory disorders including asthma, COPD and IPF (Atkinson & Senior, 2003), although there are relatively few studies reporting MMP-9 levels in airways of COPD patients.

However, substantial evidence exists in smoking related emphysema leading to the protease/anti-protease imbalance theory of its aetiology (Atkinson & Senior, 2003; Elkington & Friedland, 2006). Juanita et al reported an increase in MMP-9 activity in induced sputum from mild-to-moderate COPD patients; in addition they also suggested an aetiological protease-antiprotease imbalance in COPD (Vernooy, Lindeman, Jacobs, Hanemaaijer, & Wouters, 2004). Mercer and colleagues reported increase in both MMP-9 and TIMP-1 levels in sputum of COPD patients during acute exacerbations (Mercer et al., 2005).

In EMT, MMP-9 expression is up-regulated in epithelial cells (Atkinson & Senior, 2003) and can act as a marker of epithelial and basement membrane damage (Figure 20) (Ward et al., 2005). Type IV collagen is the major component of the “true” basement membrane (BM) and MMP-9 has the potential to digest the BM collagen and hence can assist the migration of cells through the BM into the lamina propria (Atkinson & Senior, 2003; Raghu Kalluri & Neilson, 2003; Ward et al., 2005). MMP-9 is one of the major MMPs secreted during the process of EMT (Raghu Kalluri & Neilson, 2003). Chris Ward et al reported an increased expression of MMP-9 during EMT in clinically stable lung transplant recipients (Ward et al., 2005). Tan and colleagues reported that MMP-9 mediates EMT *in vitro* in murine renal tubular cells (Tan et al.). Another study suggested that MMP-9 can mediate EMT by disrupting the E-cadherin complex in renal tubular cells hence promoting migration (Zheng et al., 2009). MMP-9 also stimulates EMT during tumor development (Orlichenko & Radisky, 2008). MMP-9 expression has also been reported in IPF and may be involved in cell migration and activation of TGF- β , hence facilitating the process of EMT in that disease (Atkinson & Senior, 2003). MMP-9 expression has also been observed in human non-small cell lung cancer and was significantly associated with its biological behaviour (Chen et al., 2008). These studies suggest that MMP-9 can play an important role in the process of EMT.

2.10.3 Vimentin

Vimentin is a 57 kDa type III intermediate filament protein normally expressed in cells of mesenchymal origin, cells undergoing EMT (Raghu Kalluri & Neilson, 2003) and also in tumour invasion (Fuchs & Weber, 1994). Vimentin increases in abundance in epithelial cells undergoing EMT suggesting a particular role in

epithelial cell migration (J. M. Lee et al., 2006). Gilles et al reported that vimentin could be functionally involved with migration of mammary epithelial cells during EMT (Gilles et al., 1999). In breast cancer, observations made both *in vitro* and *in vivo* by Kokkinos et al suggested that during cancer-associated EMT, cell intermediate filament status changes from a keratin rich network to a vimentin rich network favouring migration and increased invasion (Kokkinos et al., 2007). In another study, co-expression of vimentin and keratin intermediate filaments was associated with increased motility in human melanoma cells (Chu, Seftor, Romer, & Hendrix, 1996). Increased vimentin expression was also reported during EMT in human kidney transplant patients (Vongwiwatana, Tasanarong, Rayner, Melk, & Halloran, 2005).

Data on vimentin in lungs is very sparse. However, Borthwick et al demonstrated an increase in vimentin with subsequent decreases in airway epithelial markers like E-cadherins during the process of EMT and airway remodelling in lung transplant recipients (Borthwick et al., 2009). In a recent study, Hackett et al found increased levels of vimentin in TGF- β -induced EMT in primary airway epithelial cell cultures from patients with asthma (T. L. Hackett et al., 2009). Increased levels of anti-vimentin antibody were also observed in sera of patients with IPF (Y. Yang et al., 2002) suggesting abnormal expression of this protein in this disease.

2.10.4 (Cyto) keratins

Cytokeratins are keratin-containing intermediate filaments found in epithelial cells and are vital for regular tissue function and structure. The term “cytokeratin” was used in 1970 when keratins were first identified inside cells and then later in 2006 was technically replaced by the term “keratins” but is still widely used (Franke, Schmid, Osborn, & Weber, 1979; Schweizer et al., 2006).

Keratins are the biggest and most complex family of intermediate filaments comprising three different types (type I, type II and type III). Types I and type II are the main members, whereas type III involves vimentin, desmin, peripherin and glial fibrillary acidic protein (GFAP). Type I keratins include acidic keratin with 11 epithelial proteins (K9-K20), and also four hair keratins (Ha1-Ha4). Type II keratins

on the other hand are more basic with 8 epithelial keratins (K1-K8) identified so far (Fuchs & Weber, 1994; Schweizer et al., 2006).

Data on (cyto) keratins are widely reported in the literature on EMT. They are one group of epithelial proteins which generally decrease in abundance during EMT, but with substantial increase in mesenchymal markers (vimentin) (Kokkinos et al., 2007; J. M. Lee et al., 2006). Cytokeratins are very tightly regulated during the process of EMT and these protein changes in the cell reflect specific transcriptional activity during EMT (Savagner, 2001).

Borthwick and colleagues reported decreased expression of cytokeratin-19 with subsequent increase in the mesenchymal markers (vimentin) in epithelial cells from lung transplant recipients during TGF- β 1-induced EMT (Borthwick et al., 2009). Furthermore, in IPF a decrease in cytokeratin levels was also observed in alveolar epithelial cells during TGF- β 1-induced EMT (Brigham C. Willis et al., 2005).

2.11 EMT in the lungs

The exact role of EMT in the respiratory tract's response to injury and development of fibrosis is not clearly understood (B. C. Willis & Borok, 2007). Myofibroblasts have been implicated as key mediators of fibrosis in idiopathic pulmonary fibrosis (IPF) but the precise origin of these primary effector cells of fibrosis in the lungs is not yet established. However, there are several potential sources of fibrogenic cells within the respiratory tract: first, there may be expansion of a local fibroblast pool by in-situ proliferation in response to injury which may lead to pathological fibrosis; second, recruitment of circulating bone-marrow derived fibrocyte progenitors may occur; and third, transition of respiratory epithelial cells into a mesenchymal phenotype via EMT (Brigham C. Willis et al., 2006).

In pulmonary fibrosis it was recently reported that circulating bone-marrow derived mesenchymal progenitor cells (fibrocytes) can play an important role in the progression of pulmonary fibrosis (Strieter, Keeley, Hughes, Burdick, & Mehrad, 2009). Phillip et al demonstrated in a murine model of bleomycin-induced pulmonary fibrosis that CD45 (+) Col I (+) CXCR4 (+) fibrocytes contribute to the pathogenesis of pulmonary fibrosis (Phillips et al., 2004). Hashimoto et al

demonstrated using a chimeric mice model in which they transplanted of GFP+ (green fluorescent protein) bone marrow into a wild-type mice and then that bone marrow-derived GFP+ Col1+ cells were found in the lungs of mice exposed to bleomycin, suggesting that circulating fibrocytes may contribute to pathogenesis of pulmonary fibrosis (Hashimoto, Jin, Liu, Chensue, & Phan, 2004).

EMT has only recently been recognised in the human lung and airway, (T. L. Hackett et al., 2009; Hodge et al., 2009; Ward et al., 2005; Brigham C. Willis et al., 2006) but it is well described in lung embryogenesis, (J. M. Lee et al., 2006) metastatic malignant disease (Bjornland et al., 1999) and as part of the repair process in renal disease following tissue injury (Yanez-Mo et al., 2003). There are no reported studies in COPD associated with EMT, although Araya and colleagues suggested a key role for integrin-mediated TGF- β activation in amplifying squamous metaplasia and driving IL-1 β -dependent profibrotic mesenchymal response in smokers with COPD (Araya et al., 2007).

2.12 Histone acetylation and deacetylation in COPD

Chronic inflammatory diseases like COPD, asthma, cystic fibrosis, interstitial lung disease, inflammatory bowel disease and rheumatoid arthritis are associated with a specific pattern of inflammation, which requires a coordinate expression of a wide range of different pro-inflammatory genes coding for various inflammatory mediators leading to infiltration and activation of a panoply of inflammatory cells (Barnes, 2006a, 2006b; Barnes et al., 2005). In COPD, the specific pattern of inflammation is mainly characterized by increased numbers of luminal, sputum and BAL (bronchial alveolar lavage) neutrophils and airway wall macrophages and T-lymphocytes, predominantly cytotoxic (CD8+) cells (Barnes et al., 2005). The increased expression of these inflammatory genes is at least partly regulated by acetylation of core histones around which DNA is wound, and on the other hand these activated genes can be switched off by deacetylation of these histones (Barnes et al., 2005). Transcription of various proinflammatory genes in chronic inflammatory diseases including COPD, is regulated by transcription factors, such as nuclear factor-Kappa B (NF- κ B), activator protein-1 (AP-1) and glucocorticoid receptors as well

(which act as transcription factors when activated by glucocorticoids) (Barnes, 2006d).

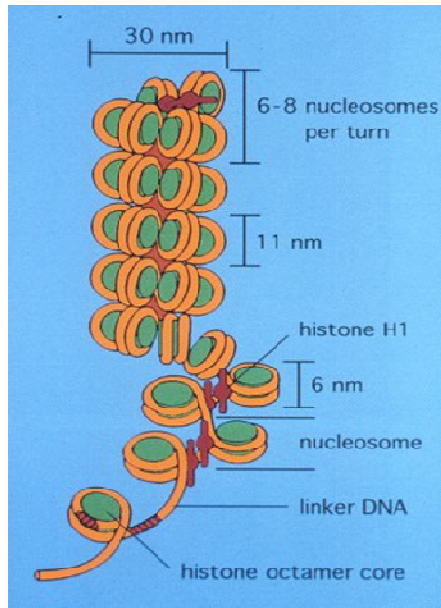
Compared to asthma, COPD responds relatively poorly to the anti-inflammatory corticosteroids. The potential molecular mechanism behind this relative corticosteroids resistance in COPD is now being revealed, as the details of the anti-inflammatory mechanisms of steroids are becoming better understood (Barnes, 2006b; Barnes & Stockley, 2005). It has been suggested that corticosteroids resistance in COPD is due to a decrease in the key nuclear enzyme histone deacetylase -2 or HDAC-2 (Barnes, 2006c; Ito et al., 2001). The exact reason behind HDAC-2 reduction in COPD is not clear, but it is suggested that increased oxidative and nitrative stress in the airways due to cigarette smoking might lead to inactivation or destruction of the enzyme (Barnes et al., 2005). Since 1960 it has been known that acetylation of DNA-associated histone proteins and remodelling of the tightly packed chromatin structure is associated with induction of a variety of genes (Littau, Burdick, Allfrey, & Mirsky, 1965). Indeed, it is now understood that histone and chromatin remodelling is central to gene expression and regulation through the process of acetylation, deacetylation and also methylation (Barnes et al., 2005; Rice & Allis, 2001).

2.12.1 Chromatin remodelling

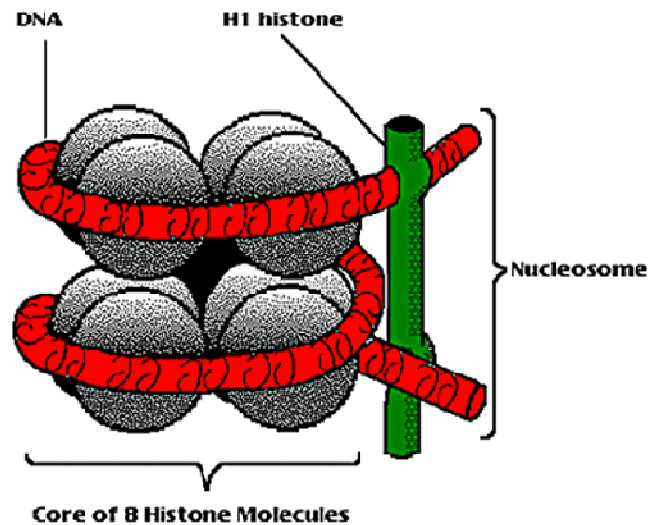
In each human eukaryotic cell of average size (say 50-100 μ in diameter), there are 46 chromosomes giving a total length of DNA per cell of 1-2 meters, packed into a nucleus millions of times smaller in diameter. A very convoluted, precise and sophisticated packing method indeed is needed to achieve this amazing phenomenon. DNA in cells exists as chromatin, a DNA-protein complex (Elliott; Peterson & Laniel, 2004). Chromatin is made up of a basic unit called a nucleosome, which further consists of 146 base pairs of genomic DNA wrapped around an octamer (8) of core histone proteins. The histone octamer consists of a central subunit of histones H3 and H4 tetramer, and two histones H2A and H2B dimers. Nucleosome are separated from each other by 10-60 base pairs of linker DNA, and the resulting nucleosomal array comprises a chromatin fibre 10 nm in diameter (Figure 2.22) (Barnes et al., 2005; Rahman, 2003).

In a resting cell, chromatin is a tight compact structure with DNA tightly wound around the core histones, which makes DNA inaccessible to various transcription factors and RNA polymerase II, which has the potential to induce messenger RNA formation. This architecture or conformation of the chromatin is described as “closed” and is associated with gene suppression. Gene transcription can only occur if the chromatin structure becomes loose and is “opened” by unwinding of the DNA wrapped around the histone proteins, so that the DNA becomes accessible to the various transcription factors and RNA polymerase II enzyme to initiate the process of transcription of respective genes (Barnes et al., 2005; Rahman, 2003).

This alteration in the chromatin architecture is known as “chromatin remodelling”, which means the effective removal of nucleosomes from the promoter site of the gene to be activated. However, it is not well understood whether a nucleosome is physically dissociated from the DNA during the whole process or just changes its attachment or is completely lost, so as to permit the transcription machinery to assemble on the promoter site of the gene to be activated. The term remodelling avoids implications of what exactly is happening in molecular terms. It is not known how many nucleosomes need to be remodelled during this gene activating procedure (Elliott; Rahman, 2003). However, it is understood that the chromatin structure is strongly controlled by post-translational modification of histones, which may involve methylation and/or phosphorylation, but the two best studied modifications are histone acetylation and histone deacetylation which involves opposing types of enzymes, respectively (de Ruijter, van Gennip, Caron, Kemp, & van Kuilenburg, 2003).



Chromatin fiber



Nucleosome

Figure 2.22: Diagrammatic representation of a chromatin fibre and a nucleosome, taken from (bricker.tcnj.edu/Amb/le8/nucleosome.jpg) and (employees.csbsju.edu/.../DNA/oldnastructure.html)

2.12.2 Histone acetylation

Acetylation is the best understood histone modifications. Transcriptionally active genes are regulated by acetylation of lysine residues on N-terminal histone tails. All core histones become acetylated, but modifications to H3 and H4 are more characterised than H2A and H2B. Hypoacetylated histones are associated with gene suppression (de Ruijter et al., 2003). Acetylation leads to the opening of the chromatin structure, allowing recruitment and activation of proinflammatory transcription factors (P. J. Barnes, 2008).

When these transcription factors are themselves activated in response to extracellular stimuli, they bind to specific sites on DNA and start interacting with various coactivator molecules (Figure 2.23) such as CREB-binding protein (cyclic AMP response element binding protein), p300, p300/CBP-associated factor (PCAF) and signal transduction activated transcription factors (STATs). These act as molecular

switches to control gene transcription. All these coactivator molecules also have intrinsic complementary histone acetyltransferase (HAT) activity (P. J. Barnes, 2008; Ogryzko, Schiltz, Russanova, Howard, & Nakatani, 1996; S. Y. Roth, Denu, & Allis, 2001). Histone acetyltransferases or HATs are the enzymes responsible for acetylation of the lysine residues on histone tails and therefore the resulting opening up of the structure of chromatin, so that acetylation, opening of chromatin and DNA-RNA transcription are complementarily regulated events (S. Y. Roth et al., 2001). In COPD, expression of pro-inflammatory genes is predominantly regulated by increased acetylation of H4 which is induced by the binding of NF- κ B and AP-1 transcription factors, which also go on sequentially to activate inflammatory mediator genes (P. J. Barnes, 2008).

In a resting cell, basic histone proteins (positively charged) are tightly associated with an acidic DNA backbone (negatively charged) thus reducing the accessibility of the DNA to transcriptional activators. Each histone protein has a long terminal rich in lysine residues, acetylation of which leads to decrease in overall positive charge of the tail, which further leads to reduced association between histone tails and DNA. It may also change the conformational state of the nucleosomes, further enhancing the accessibility to DNA of transcription factors (Rice & Allis, 2001). This further allows the binding of the TATA box-binding protein (TBP), TBP-associated factors and finally binding of RNA polymerase II enzyme leading to gene transcription. Ito and colleagues reported that there is an increase in acetylation of histones associated with the promoter region of inflammatory genes, such as IL-8 which are regulated by NF- κ B. In COPD specimens of peripheral lung; airway biopsies and alveolar macrophages the rate of acetylation increases with diseases severity (Ito et al., 2005).

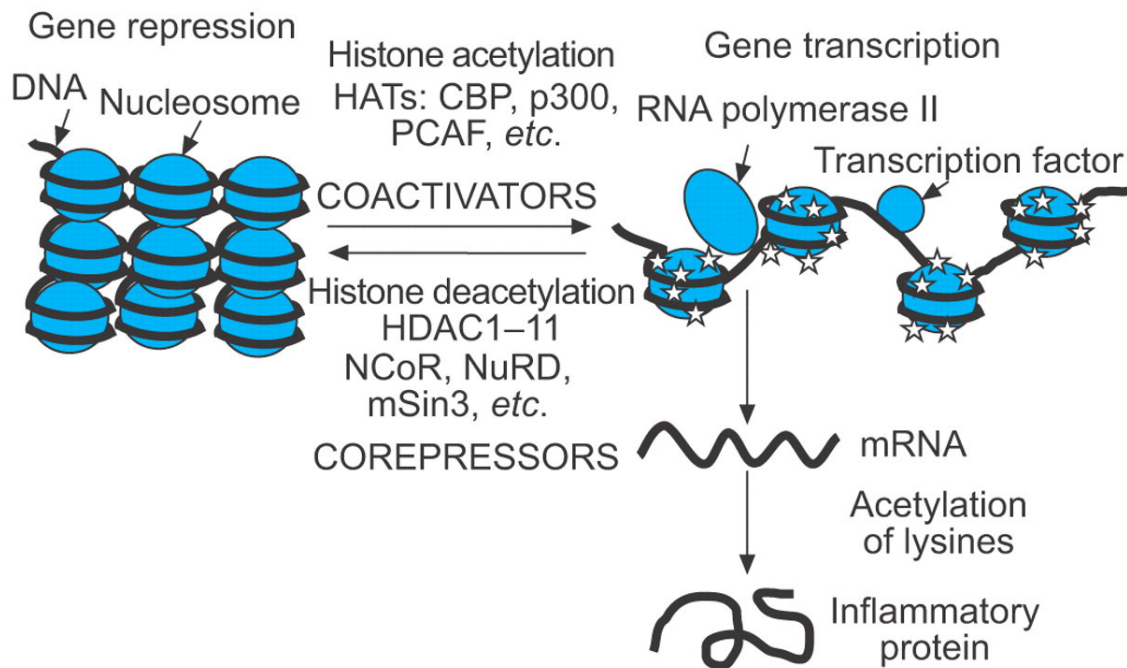


Figure 2.23: Histone acetylation leading to gene activation and deacetylation leading to gene suppression (Barnes et al., 2005).

2.12.3 Histone deacetylation

In the process of histone deacetylation, histone deacetylase enzymes (HDACs) reverse the process of acetylation by removing the acetyl groups, and are hence associated with closed conformation of the chromatin leading to gene suppression (Barnes et al., 2005). Interplay between HATs and HDACs is central to control of chromatin structure and function (Peterson, 2002). HDACs induce gene suppression by recruitment of co-repressor proteins as such nuclear receptor co-repressor (NCoR) and silencing mediator of retinoid and thyroid hormone receptors (SMRT). This leads to formation of a co-repressor complex associated with gene suppression (Figure 2.23) (Barnes, 2006b).

There are 11 HDAC isoenzymes that deacetylate histones within the nucleus and specific HDACs appear to be differentially regulated to control different set of genes. They are divided into two major classes. Class I comprises HDAC1, 2, 3, 8 and 11 whereas class II includes HDAC4, 5, 6, 7, 9 and 10. Marked reduction in HDACs has been observed in a variety of chronic inflammatory diseases (Barnes et al., 2005; de Ruijter et al., 2003; Ficner, 2009). In COPD a marked reduction in HDAC2 expression and activity has been observed, with rather less reduction in HDAC5 and HDAC8 expression, and normal expression of other HDACs. It appears that for the regulation of inflammatory genes HDAC2 appears to be of critical importance (Barnes, 2009; Ito et al., 2005).

2.12.4 HDAC2 in COPD

Ito and colleagues showed (by using an HDAC fluorescence activity assay) that there was progressive reduction in total HDAC “activity”, HDAC2 mRNA and protein expression (measured by using quantitative reverse-transcriptase–polymerase-chain-reaction (qRT-PCR) and Western blotting) in samples of peripheral lung tissue, alveolar macrophages and bronchial biopsy specimens from patients with COPD in comparison to healthy non-smokers. They also reported an increase in IL-8 mRNA expression and H4 acetylation in COPD patients (by using a chromatin immunoprecipitation assay) and interestingly there was a positive correlation between H4 acetylation and HDAC activity indicating that the balance between the two had shifted toward the hyperacetylation of the histones in the peripheral lung of patients with COPD, but no change was observed with HDAC activity in patients with pneumonia or cystic fibrosis, or patients with mild asthma, which further shows that change in HDAC activity might be specifically related to COPD. They also reported that there was a marked decrease in HDAC2 activity with lesser reduction in HDAC5 and HDAC8 in COPD (Ito et al., 2005).

In a prominent study it was reported that the decrease in HDAC activity is associated with the intensity of inflammation, as measured by IL-8 expression and the number of inflammatory cells in peripheral airways of COPD patients and also accounted for resistance to anti-inflammatory effects of corticosteroids, a typical feature associated with COPD (Barnes et al., 2005). Cigarette smoking has been considered as an main etiological factor associated to COPD, and Ito et al reported that cigarette smoking

reduced HDAC2 expression in alveolar macrophages and biopsies and enhanced the expression of inflammatory mediators (TNF- α and IL-8) and also reduced glucocorticoid responsiveness in alveolar macrophages (Barnes et al., 2005; Ito et al., 2001). Their immunohistochemical analysis suggested that HDAC2 expression is not different between non-smokers and normal lung function smokers in bronchial biopsies, and although localized to all airway cells, most intense staining was within the epithelium. However, this analysis was limited by lack of any analysis of the cellular profile of the biopsy sections. Looking now at the images they published in the paper it is quite clear that total cellularity of the lamina propria in normal lung function smokers was substantially less than in normal controls (Figure 2.24). This could lead to potential bias associated with results produced from molecular techniques and protein analysis (Ito et al., 2001). Using Western blotting they showed reduced HDAC2 protein content in bronchial biopsies from normal lung function smokers compared to normal controls (Ito et al., 2001), but as stated above, this did not take into account the overall decrease in cellularity and therefore total protein available.

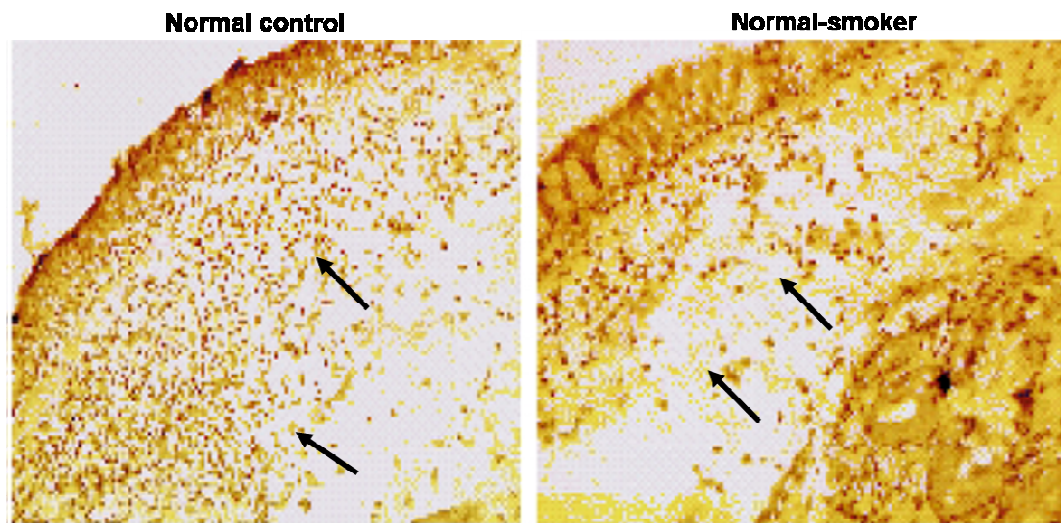


Figure 2.24: Showing decreased cellularity in lamina propria (black arrows) in a normal lung function smoker compared to a normal control, stained for HDAC2 (Ito et al., 2001).

Marwick et al reported that HAT activity is increased and HDAC2 activity is decreased in lungs of rats exposed to cigarette smoke, with increased NF- κ B activation and inflammatory gene expression (Marwick et al., 2004), but again it is difficult to know if any cell density changes were taken into account. In another study Ito and colleagues reported that inducing HDAC2 over-expression in glucocorticoid-insensitive alveolar macrophages from COPD patients restored glucocorticoid sensitivity (Ito et al., 2006). Tomita et al reported that oxidative stress has the potential to acetylate H4 in epithelial cell lines and lead to elevated levels of inflammatory cytokines such as IL-8 (Tomita, Barnes, & Adcock, 2003).

2.12.5 Mechanism of HDAC2 reduction in COPD

The reason for the decrease in total HDAC activity in COPD, especially HDAC-2 is not well understood, but evidence is emerging gradually that this may be due to increase in oxidative and nitrative stress in the lungs with COPD (Barnes et al.,

2005). Peter Barnes indicated (Figure 2.25) that cigarette smoke and inflammatory cells produce superoxide anions (O_2^-) and nitric oxide (NO), and this leads to the formation of peroxynitrite. Inducible nitric oxide synthase (iNOS) induces NO production from inflammatory cells during the process. Peroxynitrite nitrates HDAC2, possibly at tyrosine residues within the catalytic site of the enzyme, which may then obstruct enzymatic activity and may also mark the enzyme protein for ubiquitination, and so degradation proteasome which leads to a decrease in HDAC2 expression and intensification of the inflammatory response and increased resistance to corticosteroids in COPD.

HDAC activity may be restored using antioxidants, iNOS inhibitors, or by using peroxynitrite scavengers that potentially decrease tyrosine nitration. Theophylline may also act as an HDAC activator and has the potential to restore HDAC, but the mechanism behind it is not well understood (Barnes, 2006d; Barnes et al., 2005; Rahman, 2003). The oxidative pathway also has the potential to activate the phosphoinositide-3-kinase (PI3K) pathway leading to phosphorylation of serine residues, also resulting in inactivation of HDAC2 (Barnes, 2009).

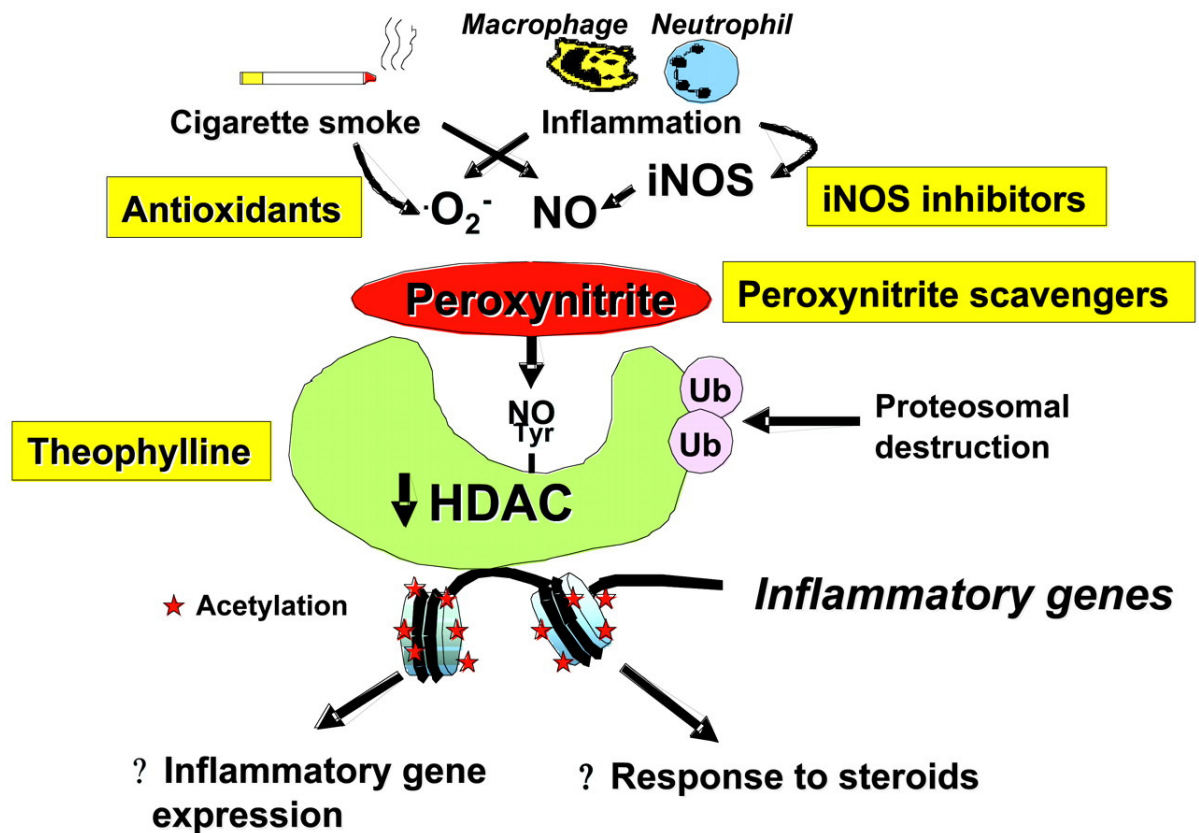


Figure 2.25: Possible mechanism of HDAC2 impairment in COPD (Barnes, 2006a).

2.13 Role of inhaled corticosteroids (ICS) in COPD

Corticosteroids or more fully glucocorticosteroids (though commonly used clinically in asthma and COPD simply as “steroids”) are considered as the most effective therapy for the treatment of non-infectious chronic inflammatory airway disorders, especially asthma. They are thought of as relatively less effective in other inflammatory disorders such as COPD, although are very widely used in this condition. Mechanistically, the main effect of steroids at the cellular level is to inhibit the expression of multiple inflammatory genes encoding for chemokines, cytokines and growth factors, activated as part of the inflammatory process (Barnes, 2006a).

As stated above, it is perhaps paradoxical that steroids are extensively used for the treatment of COPD even though their efficacy is still a matter of debate (Telenga, Kerstjens, Postma, Ten Hacken, & van den Berge). However, several studies have shown that short term use of ICS improves lung function and frequency of exacerbation in long term users (Chanez et al., 2004; Telenga et al.).

Pauwels et al in a double-blind, placebo-controlled study in 1277 subjects with moderate COPD who continued smoking, investigated the effects of budesonide 400µg, and reported that during the first six months of the study, FEV1 improved at the rate of 17 ml per year in the treatment arm, as compared with a placebo group (81 ml decline). However, from nine months of treatment to the end of treatment which was at 3 years there was no significant difference between the groups, suggesting that ICS is associated only with a short term improvement in lung function but does not appreciably affect the long-term progressive decline in lung function in patients with COPD (Pauwels et al., 1999).

A multicenter TORCH study (Toward a Revolution in COPD Health) performed with 5343 patients with COPD in 2004 evaluated the effects of fluticasone propionate combined with salmeterol (LABA, long acting β agonist) for 3 years. In the treatment arm a reduced rate of lung function decline was observed compared to the placebo group (Vestbo, 2004).

As explained above, it can be concluded that steroids do improve lung function but still today the type of inflammation and structural changes effected by ICS in COPD are not well understood, and there are very few studies reporting the effects of ICS on airway inflammation and even fewer on structural changes (airway remodelling) in COPD. Overall our knowledge is limited (Chanez et al., 2004).

Hattotuwa and colleagues reported a significant reduction in CD4/CD8 cell ratios in the epithelium, and in subepithelial mast cells in bronchial biopsies obtained from COPD patients in the active ICS arm treated with fluticasone propionate compared to placebo (Hattotuwa, Gizycki, Ansari, Jeffery, & Barnes, 2002). Reid and et al reported from observations made in bronchial biopsies and BAL from COPD

patients, that fluticasone propionate reduced BAL neutrophils and epithelial cell numbers and CD68+ macrophages, CD8+ lymphocytes and mast cells in bronchial biopsies, but interestingly noted increased neutrophils in bronchial biopsies (D. W. Reid et al., 2008). In another study, it was reported that three month treatment with fluticasone propionate significantly decreased mucosal mast cells and increased neutrophils in biopsies from COPD patients (Gizycki, Hattotuwa, Barnes, & Jeffery, 2002).

Recently, in a very interesting study, Lapperre et al reported the effects of fluticasone propionate on inflammation in airway biopsies and sputum from COPD patients, and found that fluticasone significantly decreased the number of mucosal CD3+ cells, CD4+ cells, CD8+ cells and mast cells after 3 months, with effects maintained to 30 months. They also reported that treatment with fluticasone for 30 months reduced the number of mast cells, increased number of eosinophil and increased the percentage of intact epithelium (This is the only study to my knowledge reporting effects of ICS on epithelium). There was also a decrease in sputum neutrophils, macrophages, and lymphocyte accompanied by improvements in FEV1 decline, dyspnea, and quality of life. The decrease in inflammatory cells correlated with clinical improvements. Discontinuing fluticasone for 6 months on the other hand increased CD3+ cells, mast cells, and plasma cells and thus was accompanied by deterioration in clinical outcomes (Lapperre et al., 2009).

The baseline Rbm thickness in COPD is also debatable, with some studies reporting abnormally thick Rbm and others not (Chanez et al., 2004; Liesker et al., 2009; Postma & Timens, 2006). The effects of ICS on the Rbm have not been specifically studied in COPD. Zanini and et al, (Zanini et al., 2009), however reported in a cross-sectional study that bronchial vascular remodelling may have potential for change with ICS, but we need further longitudinal studies to confirm that.

So, careful review of the literature is fairly convincing that COPD is not strictly steroid resistant as frequently stated. However, It is not clear exactly how steroids work in COPD, but in general (this is not just COPD) it is suggested that they can work in two different ways; at higher concentration they are associated with the activation of anti-inflammatory genes and at low doses they are associated with gene

suppression by recruiting HDACs to the sites of pro-inflammatory transcription, details are given below (Barnes, 2006b).

2.13.1 Gene activation by corticosteroids

At high dose the activation of anti-inflammatory genes (Figure 2.26) (Barnes, 2006b) is mainly through binding to glucocorticoid receptors (GRs) localized in the cytoplasm of the target cell; the complex acts as a transcription factor to control the transcription of several steroid responsive genes. In the cytoplasm of the cells GRs are usually attached to proteins recognized as molecular chaperons, which include heat shock-proteins-90 (hsp-90) and FK binding protein (FK or also called as FK506 is a family of proteins that have prolyl isomerase activity which suggests that they have the capacity of interconverting the cis and trans isomers of peptide bonds) (Wikipedia). These proteins when bound to the receptor prevent its nuclear localisation by covering the sites of the receptor that are essential for transportation into the nucleus (Wu et al., 2004).

Binding of corticosteroids to GR leads to changes in the receptor structure which further leads to dissociation of these inhibitory molecular chaperons proteins, by exposing the sites essential for nuclear localisation. This results in rapid transport of active GR-complex into the nucleus, where it binds to glucocorticoid response elements (GRE) in the promoter region of steroid responsive genes, including several types of anti-inflammatory genes and thus leading to increase in synthesis of anti-inflammatory proteins such as annexin-1 (lipocortin-1), secretory leuko-protease inhibitor (SLPI), IL-10, the inhibitor of NF- κ B, I κ B- α , glucocorticoid-induced leucine zipper protein, which inhibits both NF- κ B and AP-1 and mitogen-activated protein (MAP) kinase phosphatase-1, which inhibits p38 MAP kinase (Barnes, 2006b; Barnes et al., 2005).

Activation of anti-inflammatory genes by high dose of corticosteroids is associated with a selective acetylation of lysine residues 5 and 16 on H4, resulting in increased gene transcription, whereas in response to inflammatory stimuli differential acetylation of residues 8 and 12 is involved. However, the relevance of this mechanism is in doubt. It seems hard to explain the anti-inflammatory actions of

corticosteroids by inducing the transcription of a small number of anti-inflammatory genes, as high doses of corticosteroids are generally required for these types of responses, whereas on the other hand in clinical practice, corticosteroids are able to suppress inflammation at low doses (Barnes, 1998a, 2006b, 2006d, 2009; Barnes et al., 2005).

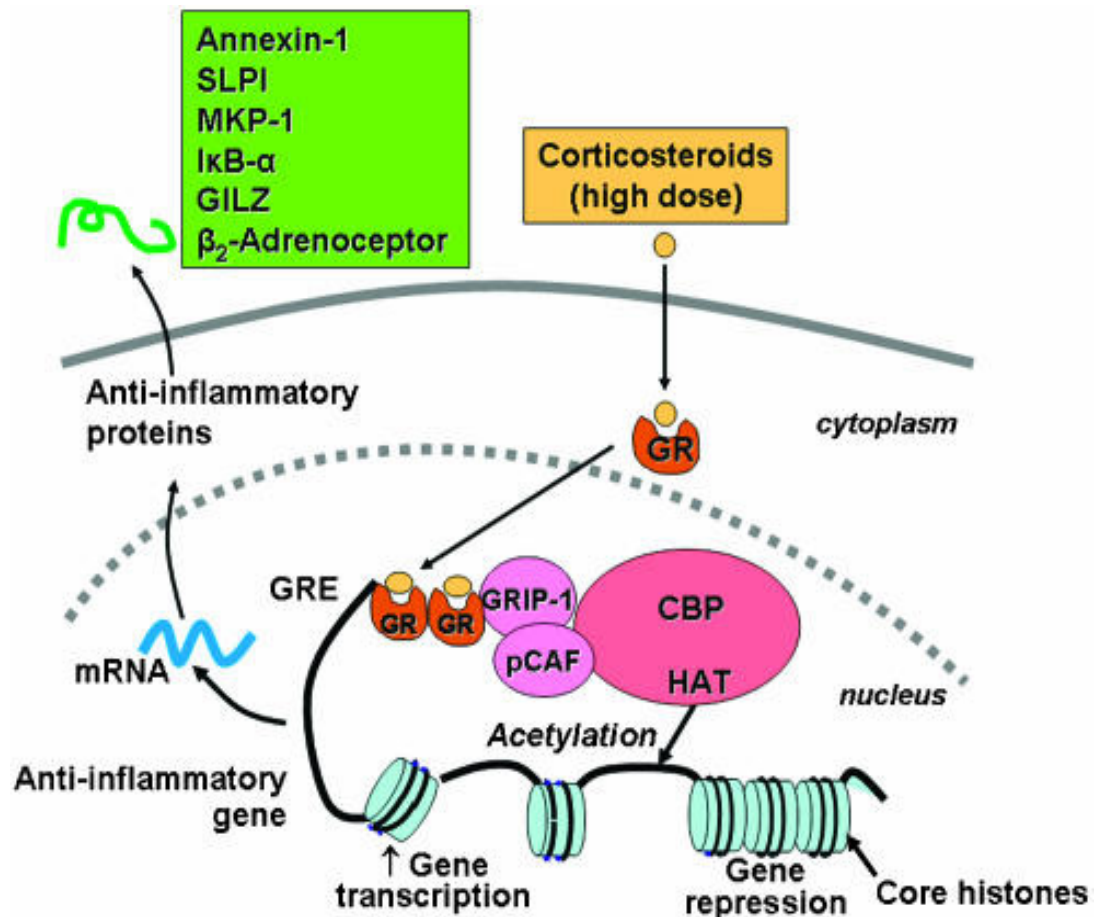


Figure 2.26: Gene activation by corticosteroids at high dose (Barnes, 2006b).

2.13.2 Gene suppression by corticosteroids

Corticosteroid at lower doses leads to suppression of inflammatory genes by recruiting HDAC2 to activated pro-inflammatory transcriptional complexes (Figure 2.27). Initially it was believed that gene suppression by corticosteroids is generally induced by binding of GR to negative GRE sites in their promoter region, but later it

was confirmed that this process is applicable to only a small number of genes, and this mechanism does not include genes encoding most inflammatory proteins (Ismaili & Garabedian, 2004). It was observed in asthma that most of the genes which are activated during the inflammatory process do not have GRE sites, but are still effectively repressed by corticosteroids (Barnes et al., 2005). There is convincing evidence now that anti-inflammatory actions of corticosteroids are due to inhibition of transcription factors such as AP-1 and NF- κ B (by inhibition of histone acetylation and stimulation of histone deacetylation), which are transcription factors actively involved in regulation of many genes coding for a host of pro-inflammatory proteins (Barnes, 2006d; Barnes & Karin, 1997).

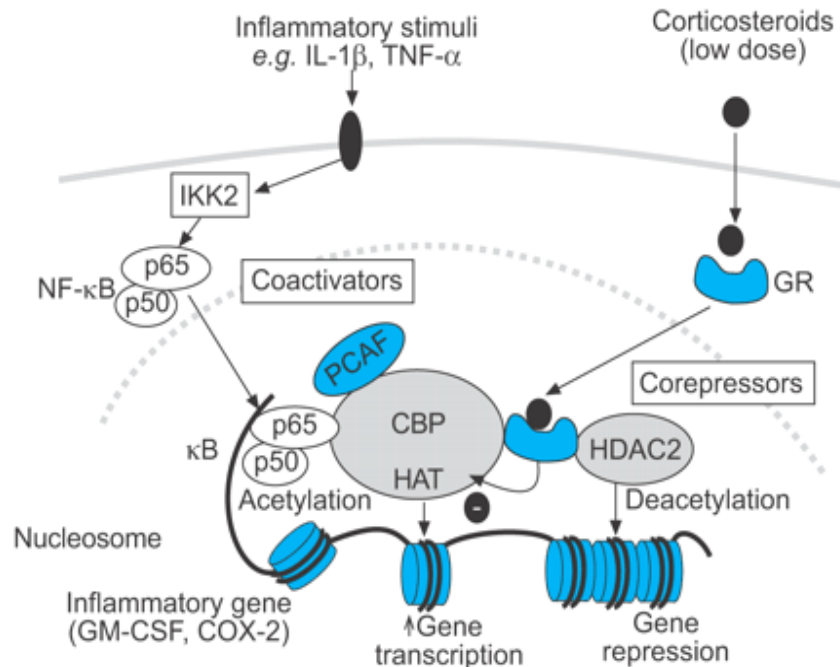


Figure 2.27: Gene suppression by corticosteroids at low dose (Barnes et al., 2005).

Inflammatory genes get activated in response to inflammatory stimuli (Figure 2.28) which may then be potentially reinforced by production of interleukin (IL)-1 β and/or tumour necrosis factor (TNF)- α which lead to activation of I- κ B kinase (IKK)2 which further activates NF- κ B, a heterodimer made up of proteins p50 and p65 (Barnes, 2006d). NF- κ B proteins move to the nucleus and bind to the specific κ B recognition sites and also bind to co-activator proteins like CBP or PCAF. All of these factors have intrinsic HAT activity, which leads to acetylation of lysine

residues in H4, and thereby augmented expression of genes encoding for inflammatory proteins, for e.g. GM-CSF, cyclo-oxygenase-2 (COX-2), and numerous cytokines, chemokines and receptors.

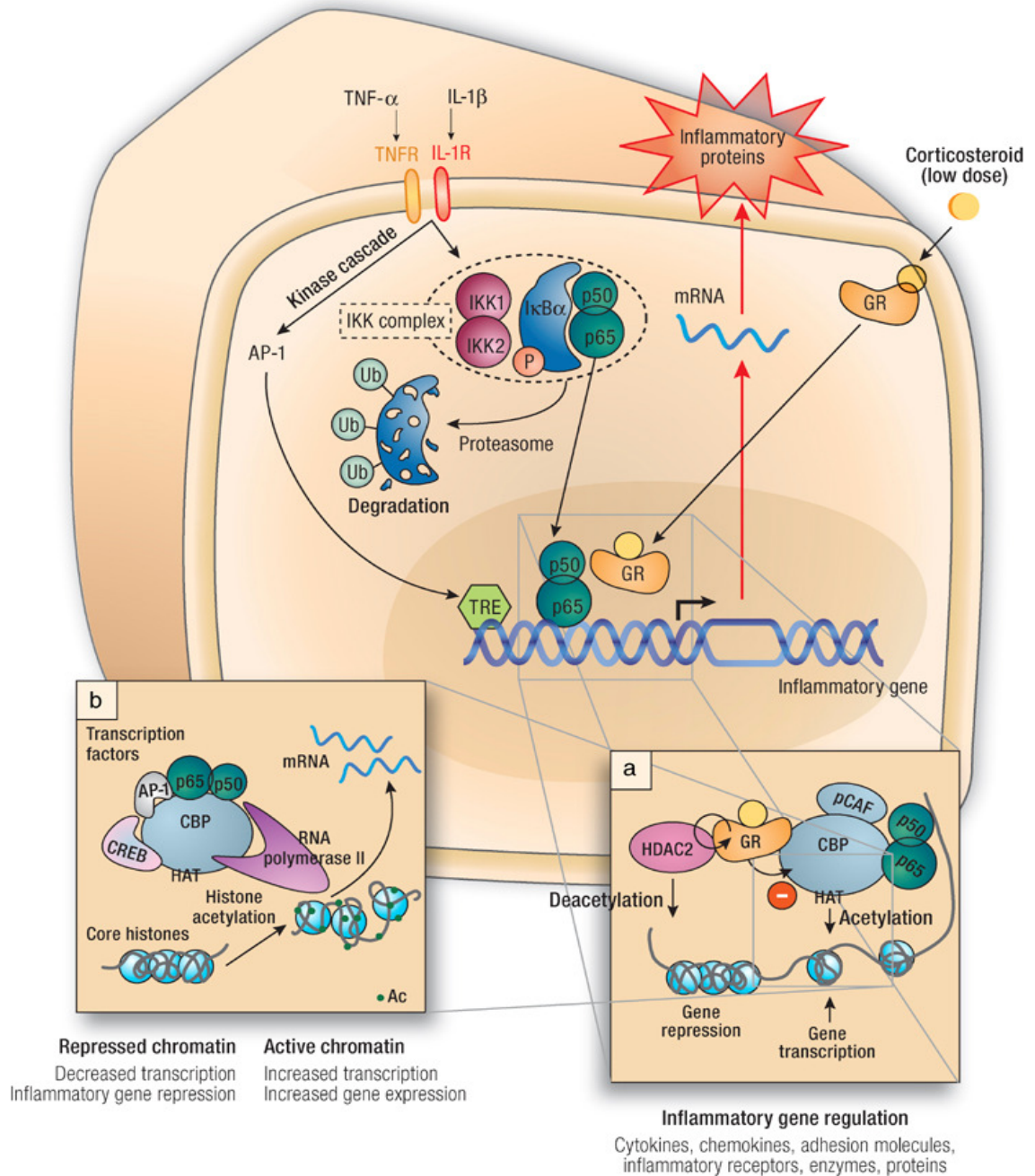


Figure 2.28: Role of transcription factors and gene suppression by corticosteroids at low dose, for details please see text (Barnes, 2006d).

Thus, this whole integrated process leads to activation of numerous inflammatory genes. As already described, corticosteroids at low concentrations activate GRs which rapidly move to the nucleus and bind to co-activators such as CBP or PCAF to directly inhibit intrinsic HAT activity, so that HDACs are recruited leading to histone deacetylation and suppression of inflammatory genes. So in very general terms, corticosteroids at low concentrations recruit HDACs to the transcription complex and convert the process of acetylation to deacetylation to suppress the transcription of inflammatory genes (Barnes, 2006a, 2006b, 2006c, 2006d; P. J. Barnes, 2008; Barnes, 2009; Barnes et al., 2005; Barnes & Karin, 1997).

2.13.3 Proposed mechanism of relative CS resistance in COPD

Even though corticosteroids have been highly effective in asthma, the inflammatory process in COPD has been at least partly and relatively resistant to the anti-inflammatory effects of corticosteroids, regardless of the fact that active airway and lung inflammation is present. Although this resistance has perhaps been over stated by some, it is true that the inflammation in COPD is not fully suppressed nor to the same extent as in asthma, by corticosteroids. (Culpitt et al., 1999; Culpitt et al., 2003; Keatings, Jatakanon, Worsdell, & Barnes, 1997; Loppow et al., 2001). Furthermore, Hogg and colleagues reported in a histological analysis of peripheral airways of patients with COPD that despite treatment with high dose steroids intense inflammatory responses still existed (James C. Hogg et al., 2004), and evidence is available that COPD has active steroid resistance mechanisms (Culpitt et al., 1999; Culpitt et al., 2003; Keatings et al., 1997; Loppow et al., 2001). Corticosteroid resistance in COPD may be explained on the basis of increased oxidative and nitrative stress due to cigarette smoking leading to impaired HDAC2 function in COPD (Figure 2.25) and resistance to corticosteroids as explained before in previous sections (Barnes, Ito, & Adcock, 2004).

The oxidative pathway also has the potential to activate the phosphoinositide-3-kinase (PI3K) pathway leading to phosphorylation of serine residues, which results in inactivation of HDAC2 (Barnes, 2009). PI3K are a family of enzymes involved in cellular functions such as cell growth and migration, inflammatory cell function, proliferation and differentiation, motility, survival and intracellular trafficking. They are divided into three different classes: the primary class reported to be associated

with impairment of steroid responsiveness in COPD is class I PI3K, including the isoforms PI3K γ and PI3K δ , and especially PI3K δ (Ito, Caramori, & Adcock, 2007).

Marwick et al investigated this system in a study designed to evaluate the role of PI3K in the development of cigarette smoke-induced steroid insensitivity in a model of wild type, PI3K γ knock out and (dead) PI3K δ kinase knock-in transgenic mice. They showed that cigarette smoke reduced HDAC2 activity and impaired steroid function in mice, which was blocked by elimination of PI3K δ kinase as shown in (dead) PI3K δ kinase knock-in transgenic mice. The overall data suggested that activation of the PI3K δ isoform might be playing an important role in the development of impaired steroid function in an oxidative environment (Marwick et al., 2009).

In another study, using peripheral lung tissue and blood monocytes obtained from patients with COPD, smokers with normal lung function and healthy never smokers, the same group reported that PI3K δ and Akt (protein kinase) phosphorylation was increased in macrophages from patients with COPD and smokers compared to controls *in vivo*. Their *in vitro* data revealed that oxidative stress-induced phosphorylation disappeared by selective inhibition of PI3K δ but not of PI3K γ . However, the study was limited by the fact that COPD subjects were also suffering from lung cancer (Marwick et al.) and material was obtained from cancer-containing lung resections. However, these studies taken together suggest that PI3K might be playing an important role in steroid unresponsiveness in COPD and PI3K δ might be a future therapeutic target.

Perhaps the important question that the Barnes group, from which these data have mainly emerged, have not asked, is how/why ICS do (undoubtedly) have some positive effects both clinically and also on inflammation in COPD airways, at least over 6-9 months, and what important elements of inflammation, and indeed remodelling could be made more responsive if the PI3K system was suppressed? In other words, if COPD really is a corticosteroid-resistant disease, what are the strategically important resistant elements of its pathology?

Chapter 3

Materials and Methods

3.1 Introduction

In preliminary observations we found that the Rbm in smokers is extensively fragmented, which can be a hallmark of EMT, as explained in previous chapters. Following our preliminary observation (Chapter 1), I designed a comprehensive cross-sectional study to test the hypotheses. I investigated current smokers with established COPD (COPD-CS) and also current smokers with normal lung function (NS), comparing them to ex-smokers with COPD (COPD-ES) and normal healthy, never-smoking controls (NC) by immuno-staining airway biopsies for different markers of EMT (S100A4, MMP-9, vimentin, cytokeratin and EGFR). To address the potential for our data to be confounded by infiltrating inflammatory cells, representative sections from the same tissue blocks were stained for neutrophil elastase (neutrophil marker), CD68 (macrophage and mature fibroblast marker) (Beranek, 2005; Inoue, Plieth, Venkov, Xu, & Neilson, 2005), CD4+/CD8+, CD19 as a B-cell marker (Bertrand et al., 1997) and CD11c for potential dendritic cells (K. F. Chung & Adcock, 2008). To confirm the extent of suppressed HDAC2 expression, airway biopsies were immuno-stained for HDAC2. In a longitudinal analysis, I used the material collected in a double blind randomised controlled trial comparing fluticasone propionate (0.5 mg/twice daily) with placebo for 6 months (D. W. Reid et al., 2008), and assessed the effects of ICS on Rbm fragmentation and HDAC2. Materials and methods for these studies are detailed in this chapter.

3.2 Subject recruitment

The study was approved by the Alfred and Royal Hobart Hospital Ethic Committees. All subjects (Table 3) gave written, informed consent prior to participation. Seventeen current smokers with established COPD (COPD-CS), 16 current smokers with normal lung function (NS), 15 ex-smokers with COPD (COPD-ES) and 15 normal healthy, never-smoking controls (NC) were recruited by advertisement in local newspapers and placement of posters in clinic waiting areas in the hospital, as

well as on the notice boards of social and Veterans clubs. Potential participants were interviewed and examined by a respiratory physician clinically, physiologically and ultimately pathologically.

Table 3: Demographic and lung function data for subjects.

Groups (numbers)	COPD-CS (n=17)	COPD-ES (n=15)	NS (n=16)	NC (n=15)
GOLD I/GOLD II [‡]	10/7	8/7	N/A	N/A
Male/female	9/8	9/6	12/4	7/8
Age (years)	61 (46-78) (p=0.001) [†]	62 (53-69) (p=0.001) [†]	50 (30-66) (p=0.31)	44 (20-68)
Smoking (pack years)	45 (18-78)	51 (18-150)	32 (10-57)	0
FEV1% predicted (Post BD) [*]	83 (66-102) (p<0.001) [†]	83 (54-104) (p<0.001) [†]	99 (78-125) (p=0.01) [†]	113 (86-140)
FEV1/FVC % (Post BD) [*]	59 (46-68) (p<0.001) [†]	57 (38-68) (p<0.001) [†]	77 (70-96) (p=0.22)	82 (71-88)

Data expressed as median and range

[†]Significance difference from NC

^{*} Post BD values after 400µg of salbutamol

[‡] Diagnosis of COPD was made according to GOLD guidelines (GOLD, 2007)

3.2.1 Exclusion criteria

1. Subjects with a history suggestive of asthma, which includes symptoms in childhood, related atopic disorders, eczema or hay fever, substantial day-to-day variability or prominent nocturnal symptoms, or a history of wheeze rather than progressive breathlessness and any who had previously used ICS (oral or inhaled) were excluded.
2. Significant uncontrolled comorbidities such as diabetes, angina or cardiac failure, and other coexisting respiratory disorders including pulmonary fibrosis, lung cancer and bronchiectasis.

3. Subjects with inability to give written informed consent were also excluded.

3.2.2 Inclusion criteria

1. Current-smokers with COPD aged at least 40 years with smoking history equal to or more than 15 pack-years and subsequently obtained BAL fluid had to be free of bacterial colonisation; FEV1 40% to 80% predicted, with FER (ratio of FEV1 to FVC) $\leq 70\%$ post bronchodilator with definite scalloping out of the descending limb of flow-volume loop on spirometry. COPD ex-smokers with at least six months of smoking cessation were included.
2. Normal healthy never smoking controls and current smokers with normal lung function recruited also underwent bronchoscopic examination and physiological evaluation. They were at least 18 years old and FEV1/FVC ratio of 70% or higher and FEV1% predicted of 80% or higher.
3. None of the never-smoking controls individuals had any history of respiratory illness or smoking. For normal lung function current smokers the inclusion criteria were; a minimum 10 pack-year history of cigarette smoking with spirometry within normal limits (FEV1 (forced expiratory volume) $>80\%$ of predicted, and FEV1/FVC (forced vital capacity) $>70\%$) and no scalloping out of the expiratory descending limb of the flow-volume curve, suggesting small airway dysfunction.

3.3 Bronchoscopy

Fiberoptic bronchoscopy was performed, subjects were pre-medicated with nebulized salbutamol (5 mg) approximately 15–30 min before the bronchoscopic examination and sedation was achieved with intravenous administration of midazolam (3–10 mg) and fentanyl (25–100 μ g) to COPD current smokers and ex-smokers and normal healthy non-smokers and smokers with normal lung function. Lignocaine (4%) was applied topically to the nose, pharynx and larynx, and 2 % lignocaine was administered below the cords in 2 ml aliquots as required up to a maximum dose of 6ml. Subjects were monitored by pulse oximetry and administered oxygen during the

procedure. Endobronchial biopsies were taken from subsegmental carinae of the right lower pulmonary lobe of each patient, using alligator forceps (FB-15C; Olympus, Tokyo, Japan). At each fiberoptic bronchoscopy performed, up to eight good biopsy specimens were obtained.

3.4 Processing of biopsies

Two biopsies were immersed with ornithine carbamyl transferase (OCT) and snap frozen in liquid nitrogen-chilled isopentane slurry, and stored at -80°C for immunohistochemistry and two more were snap frozen in liquid nitrogen for molecular studies (not presented here). The other four were fixed in 10% neutral buffered formalin for two hours, and then transferred into 50% ethanol until they were processed using a Leica ASP 200 tissue processor. Fixed tissue blocks were progressively dehydrated in graded ethanol followed by clearing in xylene before being infiltrated with wax under vacuum pressure. Two 3 µm paraffin embedded sections, separated by at least 50 microns, were cut for staining and mounted on a slide (Figure 3.1).

3.5 Immunostaining with staining antibodies used

Sections were stained with the following monoclonal antibodies: anti-MMP-9 (R&D systems, cat no. MAB911, Gympsea, NSW, Australia), anti-EGFR (Chemicon, cat no. CBL417, Billerica, United States), anti-S100A4 polyclonal antibody (Dako, cat no. A5114, Glostrup, Denmark), anti-Cd11c monoclonal antibody (Abcam, cat no. ab52632, Waterloo, NSW, Australia), anti-CD4/CD8 monoclonal antibodies (Novocastra, cat no. NCL-CD4-IF6 / cat no. NCL-CD8-4B11, Newcastle Upon Tyne, UK) were used for CD4/CD8 T lymphocytes, anti-CD68 monoclonal antibody for macrophages (Dako, cat no. M0814, Glostrup, Denmark) and mature fibroblasts, anti-CD19 monoclonal antibody for B-cells (Abcam, cat no. Ab31947, Waterloo, NSW, Australia), neutrophil elastase, a monoclonal antibody for neutrophils (Dako, cat no. Ab31947, Glostrup, Denmark) and for HDAC2, sections were stained with monoclonal antibody: anti-HDAC2 (Abcam cat no.ab12169, Waterloo, NSW, Australia), together with a horseradish peroxidase (HRP) conjugated DAKO Envision plus reagent for secondary antibody binding and colour resolution using diaminobenzidine (DAB). Double staining with vimentin and cytokeratin was

elaborated using an anti-vimentin monoclonal antibody (Dakocytomation, cat no. M 7020 clone 3B4 at 1:1000 for two hours, Glostrup, Denmark) and a pan-cytokeratin monoclonal antibody for cytokeratin (cytokeratin AE1/AE3 at 1/40 in diluent Abcam ab 27988, Waterloo, NSW, Australia) and then secondarily bound using Dakocytomation “Real” (cat no K5005) alkaline phosphatase reagents and visualised using Dakocytomation liquid permanent red (Cat no. K0640, Glostrup, Denmark). The primary antibody was replaced using a species appropriate IgG1 at equivalent dilutions and conditions on a sequential section as negative controls. Details of general immunostaining procedure for different tissue markers used are given below. All tissues were stained with Haematoxylin before immunostaining. All monoclonal and polyclonal antibodies were titrated to maximal sensitivity and to minimise non-specific staining using standard methods (Immunocytometry solutions and Histo-techniques)

3.5.1 Measurements and biopsy analysis

Computer-assisted image analysis was performed with a Leica DM 2500 microscope (Leica Microsystems, Germany), Spot insight 12 digital camera and Image Pro V5.1 (Media Cybernetics, USA) software (Figure 3.2). On each slide two biopsy sections were fixed (Figure 3.1). Firstly, as many pictures as possible were taken of the tissue from the area of interest (for this study it was mainly epithelium, Rbm and lamina propria) avoiding overlapping of tissue. All image analyses were done using the above described image analyser. Five randomly selected images from the total number taken were used for desired measurements. Cellular and other changes, e.g. clefts/cracks in the Rbm, (Figure 3.3) were normalised for purposes of comparisons in terms of the total length of basement membrane assessed. The length of the individual “clefts” was measured, summed and results expressed as percentage of the total Rbm length to give a comparable measure of the degree of Rbm fragmentation for each individual. EGFR was measured as percentage of epithelium stained for EGFR over total basement membrane length. Using the image analyser HDAC2 positive and total number of cells were counted up to 50 μ deep into the lamina propria and results presented as cells per sqmm of lamina propria and in the epithelium HDAC2 was measured as percentage of epithelium stained for HDAC2 over total basement membrane length. All slides were coded and randomised by an

independent person and then counted in a single batch by me blinded to subject and diagnosis, with quality assurance on randomly selected slides provided by a professional academic pathologist (Professor H.K. Muller).

3.5.2 Statistical analyses

Results are presented as medians and ranges. A non-parametric ANOVA (Kruskal Wallis Test) was undertaken, and specific group differences then explored using the Mann Whitney U test. Associations between variables were assessed using Spearman's rank test. Statistical analyses were performed using SPSS 15.0 for Windows, 2003, with a two-tailed P -value ≤ 0.05 being considered statistically significant. Wilcoxon two related-samples test was used to test the effect of ICS and placebo in the longitudinal study. Associations between variables were assessed using Spearman's rank test. Linear regression analysis for potential confounders was undertaken and differences between groups in gender balance, age, and atopy were found to be non-contributory.

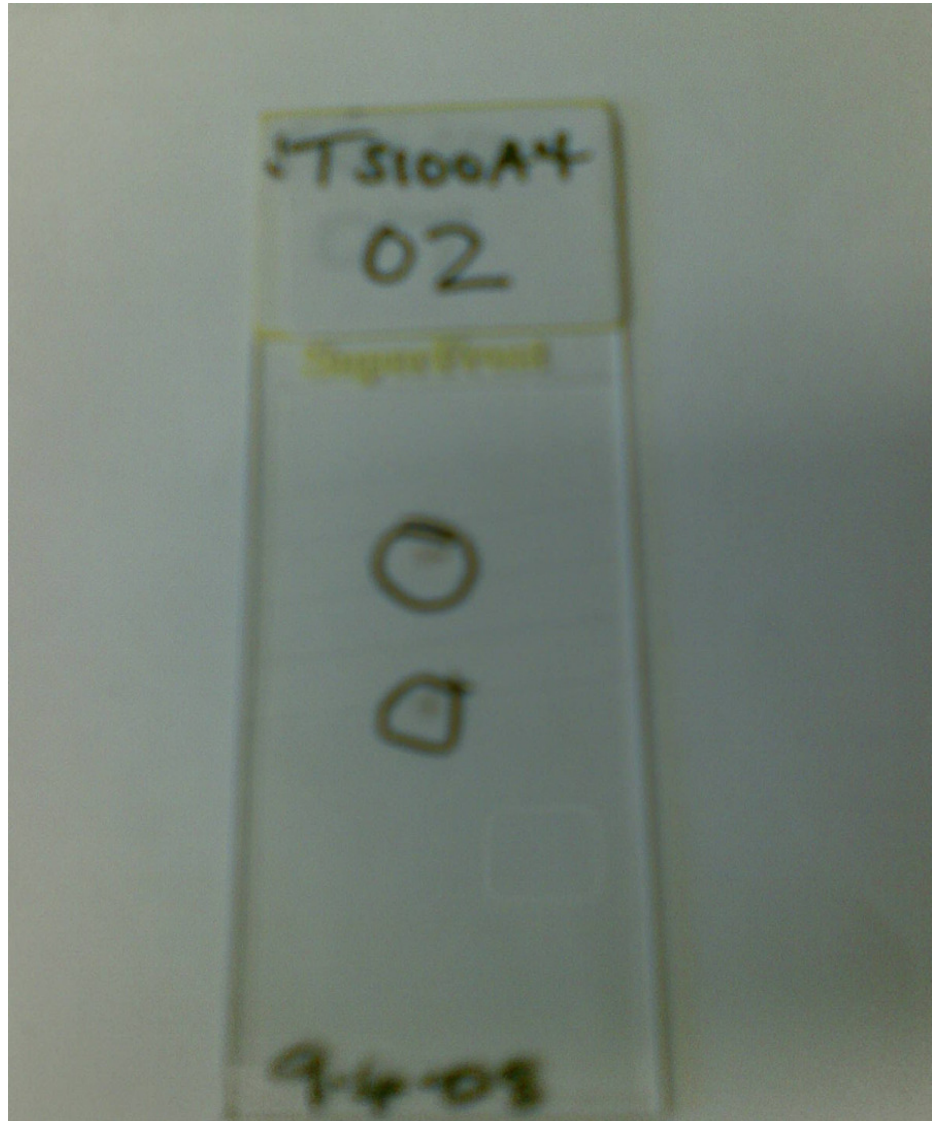


Figure 3.1: Showing two biopsy sections fixed on a slide with black circles around them.

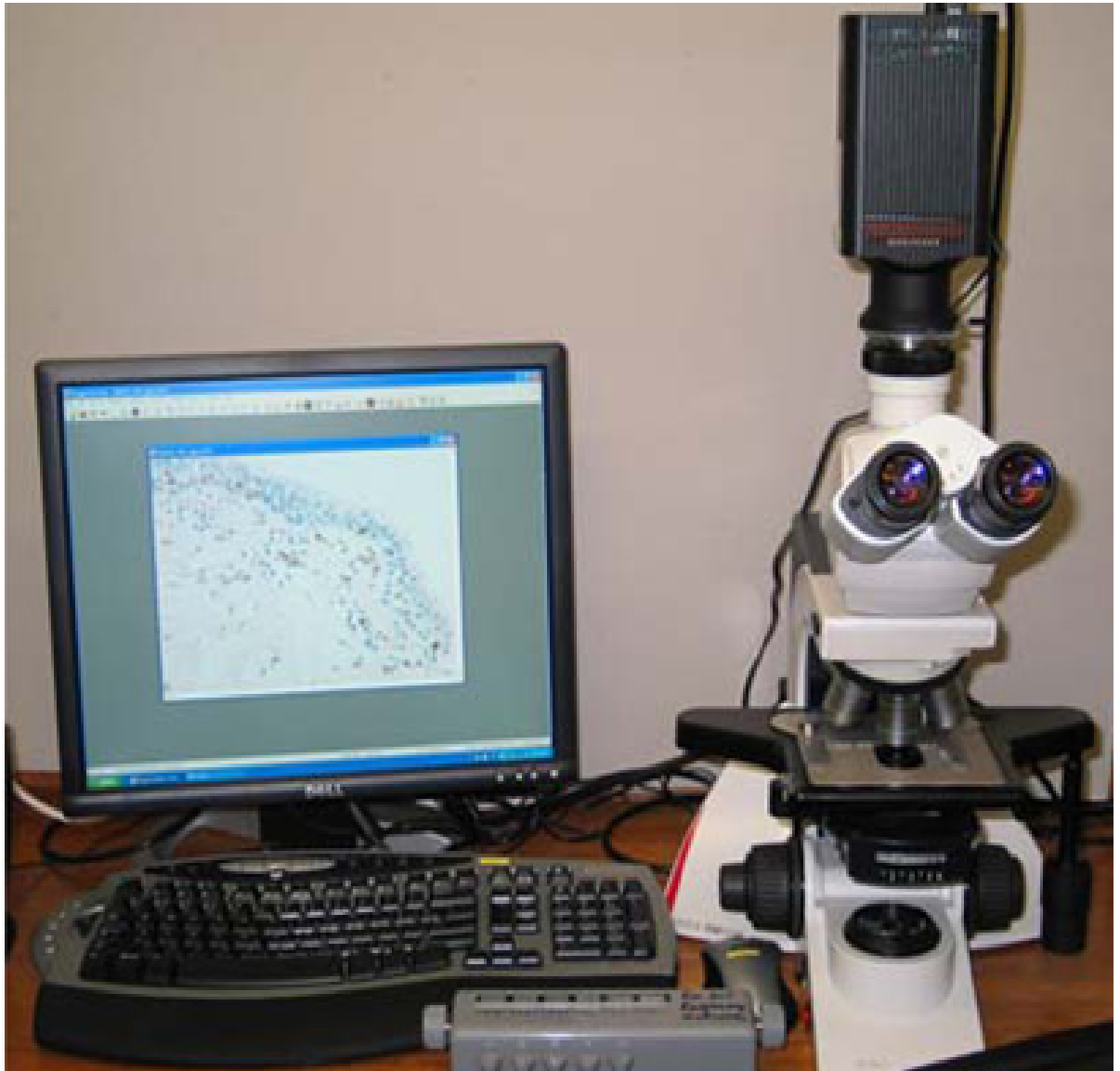


Figure 3.2: Computer-assisted image analyser (Leica DM 2500 LB microscope, Leica Microsystems, Germany; Dage-MTI DC200 one-chip video camera; Image Pro V4.5, media Cybernetics, USA software)

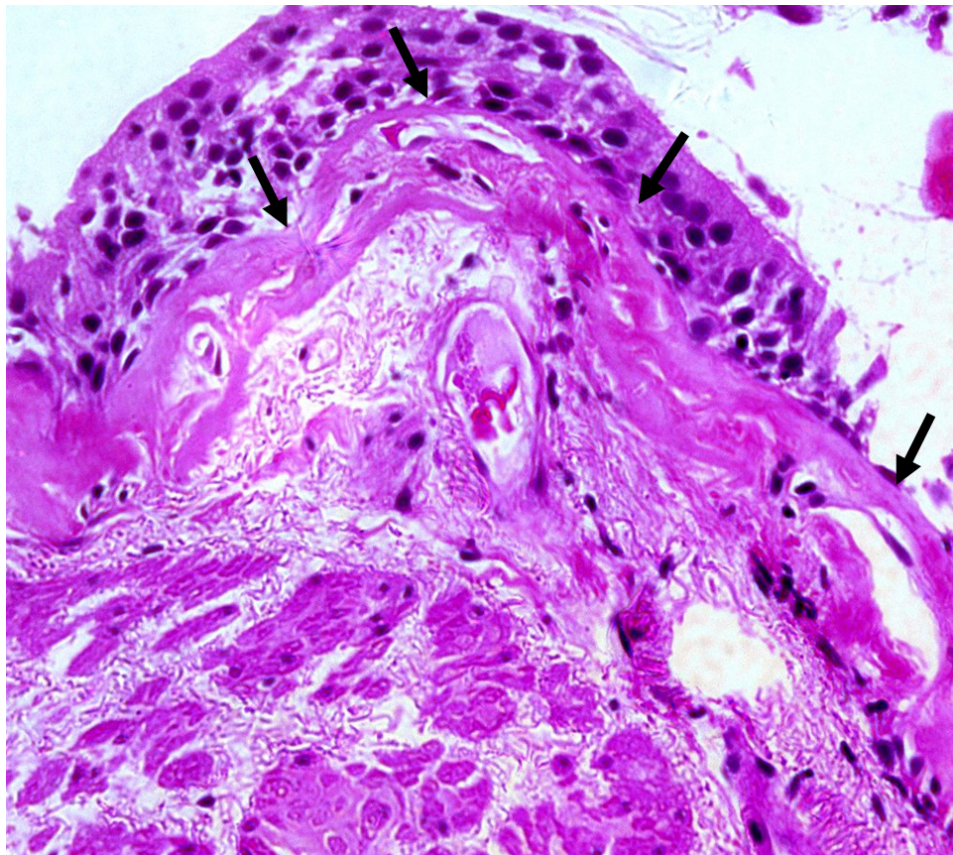


Figure 3.3: Bronchial biopsy specimen from a COPD current smoker, black arrows showing Rbm fragmentation with many “clefts” evident which frequently contain cells. Stain: haematoxylin and eosin (H & E). Original magnification, x630

3.5.3 MMP-9 immunostaining procedure

1. Paraffin blocks were sectioned at 3 microns after cooling in a -20 freezer or on ice blocks for 5-10 minutes.
2. Two sections separated by 40-50 microns (approximately 10 sections) were placed on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade).
3. The slides were dried for 2 hours in slide drying oven.
4. Slides were sorted out and had positive tissue control and a negative reagent control for each section being stained.
5. Circles were made at the back of the slide around the sections with xylene resistant pen and labelled with date of staining.
6. Sections were dewaxed in "xylene" 2 x 5 mins each in fume hood.
7. Then hydrated to water using 100% ethanol followed by 95% then 70%, 3 minutes each change.
8. Sections were rinsed well in running tap water (2 minutes).
9. Then placed in 3% H₂O₂ in distilled water for 15 minutes.
10. Washed in running water for 2 minutes.
11. Placed in 500ml of dist water in bottom of pressure cooker.
12. Added 500ml of Dako S1700 solution from the fridge, in plastic incubation vessel (or diluted to concentration 1 in 10 and reused).
13. Slides were placed in S1700 solution and then heated in pressure cooker for 6 minutes on high then 14 minutes on about 6.5 to maintain steam.
14. Allowed pressure cooker to cool and then removed the lid and placed vessel in the running water until the solution had reached 35 degrees C. Then placed sections in running water for 1 minute.
15. Covered slides with PBS pH 7.4.
16. Applied primary antibody to sections for appropriate time at appropriate concentration and temperature (1:50 for 2 hours). Also applied negative control sera (normal sera from same species as primary antibody, mouse IgG1) to control section and if possible used reagent control antibody that is

well demonstrated in the tissue under investigation. Made primary antibody using Dako diluent.

17. Washed sections well using PBS 3 x changes 5 mins each change.
18. Applied DAKO Envision + (Dako k4001) reagent to sections for 30 mins.
19. Washed sections well using PBS 3 x changes 5 mins each change.
20. Made DAKO DAB plus solution (K3468) using 20 micro litres of DAB to 1 ml of substrate buffer.
21. Applied DAB to sections for 10 mins.
22. Washed sections well using PBS 5 mins.
23. Sections were rinsed in running water.
24. Sections were placed in Mayer's haematoxylin to elaborate nuclei for 1 min.
25. Then rinsed in running water.
26. Placed in approx 400ml of water with 6-8 drops of ammonia 30 seconds.
27. Rinsed well in running water.
28. Then Dehydrated sections in clean fresh 95% ethanol, and then changed twice with 100% ethanol (2 minutes each change) (made sure that 95% is not pink, if so, it was changed).
29. Cleared in three changes of xylene (2 minutes each change) and mounted sections in Depex (or similar).
30. Then finally coverslipped using machine with 54 mm coverslips.
31. Dried on hotplate overnight.

3.5.4 EGFR immunostaining procedure

1. Paraffin blocks were sectioned at 3 microns after cooling in a -20 freezer or on ice blocks for 5-10 minutes.
2. Two sections separated by 40-50 microns (approximately 10 sections) were placed on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade).
3. The slides were dried for 2 hours in slide drying oven.
4. Sections were dewaxed in "xylene" 2 x 5 mins each in fume hood.
5. Then hydrated to water using 100% ethanol followed by 95% then 70%, 3 minutes each change.
6. Sections were rinsed well in running tap water (2 minutes).
7. Then placed in 3% H₂O₂ in distilled water for 15 minutes.
8. Washed in running water 2 minutes.
9. Rinsed in working phosphate buffered saline (diluted from Tubbs stock buffer 1 in 25) 2 mins.
10. Applied primary antibody to sections (chemicon CBL417 clone F4) 1/40 1hr made up using diluent with 3% BSA. Also applied negative control sera (normal sera from same species as primary antibody) to control section and if possible used reagent control antibody which was well demonstrated in the tissue under investigation.
11. Washed sections well using PBS 3 x changes 5 mins each change.
12. Applied DAKO Envision + (Dakok4001) reagent to sections for 30 mins.
13. Washed sections well using PBS 3 x changes 5 mins each change.
14. Made DAKO DAB plus solution (K3468) using 20 micro litres of DAB to 1 ml of substrate buffer.
15. Applied DAB to sections for 10 mins.
16. Washed sections well using PBS 5 mins.
17. Rinsed sections in running water.
18. Sections were placed in Mayers haematoxylin to elaborate nuclei, 2 mins (or in Harris's 2 mins then differentiated with three dips in 1% acid alcohol).
19. Rinsed in running water again.

20. Placed in approx 400ml of water with 6-8 drops of ammonia 30 secs.
21. Rinsed well in running water.
22. Dehydrated in 95% ethanol then changed twice in 100% ethanol (2 minutes each change).
23. Cleared in three changes of xylene (2 minutes each change) and sections were mounted in Depex (or similar).
24. Then finally coverslipped using machine with 54 mm coverslips.
25. Dried on hotplate overnight.

3.5.5 S100A4 immunostaining procedure

1. Paraffin blocks were sectioned at 3 microns after cooling in a -20 freezer or on ice blocks for 5-10 minutes.
2. Two sections separated by 40-50 microns (approximately 10 sections) were placed on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade).
3. The slides were dried for 2 hours in slide drying oven.
4. Sections were dewaxed in "xylene" 2 x 5 mins each in fume hood.
5. Then hydrated to water using 100% ethanol followed by 95% then 70%, 3 minutes each change.
6. Sections were rinsed well in running tap water (2 minutes).
7. Then placed in 3% H₂O₂ in distilled water for 15 minutes.
8. Washed in running water 2 minutes.
9. Slides were placed in pressure cooker 1 for 6 minutes on high then 15 minutes at about 6.5 on dial till just expelling steam using: 500ml of s1700 dako buffer solution with 500mls of distilled water in pressure cooker.
10. Washed in running tap water for 2 minutes.
11. Covered slides with PBS pH 7.4 for 1 minute.
12. Applied primary antibody to sections for appropriate time (90 mins rt) at appropriate concentration and temperature. Also applied negative control sera (normal sera from same species as primary antibody) to control section and if possible used reagent controlled antibody that was well demonstrated in the tissue under investigation, (S100 A4 (Dako polyclonal antibody code A 5114 protein concentration 0.72g/l) at dilution of 1/2500 (2.88×10^{-4} g/l) made from stock of 1 in 100 diluted in dako diluent and normal rabbit immunoglobulin as negative control at dilution of 1/70000 (2.85×10^{-4} g/l) (from stock Dako X0903 protein concentration 20g/l).
13. Washed sections well using PBS 3 x changes 5 mins each change.
14. Applied DAKO Envision + (rabbit polyclonal) (Dako K4003) reagent to sections for 30 mins.
15. Washed sections well using PBS 3 x changes 5 mins each change.

16. Made DAKO DAB plus solution (K3468) using 20 micro litres of DAB to 1 ml of substrate buffer.
17. Applied DAB to sections for 10 mins.
18. Washed sections well using PBS 5 mins.
19. Rinsed sections in running water.
20. Sections were placed in Mayer's haematoxylin to elaborate nuclei, 2 mins (or in Harris's 2 mins then differentiated with three dips in 1% acid alcohol).
21. Rinsed in running water.
22. Placed in approx 400ml of water with 6-8 drops of ammonia 30 secs.
23. Rinsed well in running water.
24. Dehydrated in 95% ethanol then changed twice in 100% ethanol (2 minutes each change).
25. Cleared in three changes of xylene (2 minutes each change) and mount sections in Depex (or similar).
26. Then finally coverslipped using machine with 54 mm coverslips.
27. Dried on hotplate overnight.

3.5.6 Vimentin immunostaining procedure

1. Paraffin blocks were sectioned at 3 microns after cooling in a -20 freezer or on ice blocks for 5-10 minutes.
2. Two sections separated by 40-50 microns (approximately 10 sections) were placed on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade).
3. The slides were dried for 2 hours in slide drying oven.
4. Sections were dewaxed in "xylene" 2 x 5 mins each in fume hood.
5. Then hydrated to water using 100% ethanol followed by 95% then 70%, 3 minutes each change.
6. Sections were rinsed well in running tap water (2 minutes).
7. Slides were placed in pressure cooker¹, for 6 minutes on high then 15 minutes at about 6.5 on dial till just expelling steam using: 500ml of Citrate Buffer pH6 solution with 500mls of distilled water in pressure cooker.
8. Washed in running tap water for 2 minutes.
9. Applied Dako monoclonal anti-vimentin antibody at 1/1000 for 2 hours at room temp (diluted in Dako diluent).
10. Washed three times with tris buffer for three minutes each.
11. Covered all sections in link reagent (Dako REAL K5355 or K5005), for 20mins at room temperature (RT).
12. Washed three times with tris buffer for three minutes each.
13. Incubated in REAL streptavidin (also known as AP enzyme enhancer) (Dako K5355) with ALK phos conjugate for twenty minutes at RT.
14. Washed three times with tris buffer for three minutes each change.
15. Made up chromogen by adding 20 microlitres of chromogen to each ml of substrate and then incubate sections for ten minutes.
16. Washed in running tap water for 2 minutes.
17. Sections were placed in Mayers haematoxylin to elaborate nuclei, 2 mins (or in Harris's 2 mins then differentiated with three dips in 1% acid alcohol).
18. Rinsed in running water.
19. Placed in approx 400ml of water with 6-8 drops of ammonia for 30 secs.

20. Rinsed well in running water.
21. Dehydrated on hotplate.
22. Cleared in three changes of xylene (2 minutes each change) and mounted sections in Depex (or similar).
23. Then finally coverslipped using machine with 54 mm coverslips.
24. Dried on hotplate overnight.

3.5.7 S100A4 and vimentin double- immunostaining procedure

1. Paraffin blocks were sectioned at 3 microns after cooling in a -20 freezer or on ice blocks for 5-10 minutes.
2. Two sections separated by 40-50 microns (approximately 10 sections) were placed on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade).
3. The slides were dried for 2 hours in slide drying oven.
4. Sections were dewaxed in “xylene” 2 x 5 mins each in fume hood.
5. Then hydrated to water using 100% ethanol followed by 95% then 70%, 3 minutes each change.
6. Sections were rinsed well in running tap water (2 minutes).
7. Slides were placed in pressure cooker¹, for 6 minutes on high then 15 minutes at about 6.5 on dial till just expelling steam using: 500ml of citrate buffer pH6 solution with 500mls of distilled water in pressure cooker.
8. Washed in running tap water for 2 minutes.
9. Applied Dako monoclonal anti-vimentin M7020 clone 3B4 antibody at 1/1000 for 2 hours at room temp (diluted in Dako diluent).
10. Washed three times with tris buffer for three minutes each.
11. Covered all sections in link reagent (Dako REAL K5355 or K5005), for 20 minutes RT.
12. Washed three times with tris buffer for three minutes each.
13. Incubated in REAL streptavidin (also know as AP enzyme enhancer) (Dako K5355) with ALK phos conjugate twenty minutes room temperature.
14. Washed three times with tris buffer for three minutes each change.
15. Made chromogen by adding 20 microlitres of chromogen to each ml of substrate and then incubated sections for ten minutes.
16. Washed in running tap water for 2 minutes.
17. Placed in 3% H₂O₂ in distilled water for 15 minutes.
18. Washed in running tap water 2 minutes.
19. Applied primary antibody to sections for appropriate time (60 mins RT) at appropriate concentration and temperature. Also applied negative control sera

(normal sera from same species as primary antibody) to control section and if possible used reagent control antibody which was well demonstrated in the tissue under investigation. Use S100 A4 (Dako polyclonal antibody code A 5114 protein concentration 0.72g/l) at dilution of 1/3000 (2.4×10^{-4} g/l) made from stock of 1 in 100 diluted in dako diluent Used normal rabbit immunoglobulin as negative control at dilution of 1/70000 (2.9×10^{-4} g/l) (from stock Dako X0903 protein concentration 20g/l).

20. Washed sections well using PBS pH 7.4 3 x changes 5 mins each change.
21. Applied DAKO Envision + (rabbit polyclonal) (Dako K4003) reagent to sections for 30 mins.
22. Washed sections well using PBS 3 x changes 5 mins each change.
23. Made up DAKO DAB plus solution (K3468) using 20 micro litres of DAB to 1 ml of substrate buffer.
24. Applied DAB to sections for 10 mins.
25. Rinsed sections in running water.
26. Placed sections in Mayers haematoxylin to elaborate nuclei, 2 mins (or in Harris's 2 mins then differentiate with three dips in 1% acid alcohol).
27. Rinsed in running water.
28. Placed in approx 400ml of water with 6-8 drops of ammonia 30 secs.
29. Rinsed well in running water.
30. Dehydrated on hotplate.
31. Cleared in three changes of xylene (2 minutes each change) and mounted sections in Depex (or similar).
32. Then finally coverslipped using machine with 54 mm coverslips.
33. Dried on hotplate overnight.

3.5.8 Vimentin and cytokeratin double-immunostaining procedure

1. Paraffin blocks were sectioned at 3 microns after cooling in a -20 freezer or on ice blocks for 5-10 minutes.
2. Two sections separated by 40-50 microns (approximately 10 sections) were placed on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade).
3. The slides were dried for 2 hours in slide drying oven.
4. Sections were dewaxed in "xylene" 2 x 5 mins each in fume hood.
5. Then hydrated to water using 100% ethanol followed by 95% then 70%, 3 minutes each change.
6. Sections were rinsed well in running tap water (2 minutes).
7. Placed in 3% H₂O₂ in distilled water for 15 minutes.
8. Washed in running tap water 2 minutes.
9. Slides were placed in pressure cooker¹, for 6 minutes on high then 6 minutes at about 6.5 on dial till just expelling steam using: 500ml of citrate buffer pH6 solution with 500mls of distilled water in pressure cooker.
10. Allowed to cool to below 35 degrees C before washing in running tap water 2 minutes.
11. Washed sections well using PBS pH 7.4.
12. Applied Dako monoclonal anti-vimentin M7020 clone 3B4 antibody at 1/500 for 2 hours 20 mins at room temp (diluted in Dako diluent).
13. Washed sections well using PBS pH 7.4 3x 3mins each change.
14. Applied Dako Envision + (monoclonal) (Dako K4001) reagent to sections for 30 mins.
15. Washed sections well using PBS 3 x changes 5 mins each change.
16. Made Dako DAB plus solution (K3468) using 20 micro litres of DAB to 1 ml of substrate buffer.
17. Applied DAB to sections for 10 mins.
18. Rinsed sections in running water.
19. Placed sections in Harris's haematoxylin to elaborate nuclei, 5 mins then differentiated with three dips in 1% acid alcohol.

20. Rinsed in running water.
21. Placed in approx 400ml of water with 6-8 drops of ammonia 30 secs.
22. Rinsed well in running water and check nuclei (adjusted further if necessary).
23. Washed with tris buffer for three minutes.
24. Applied primary antibody (cytokeratin AE1/AE3 at 1/40 in diluent Abcam ab 27988) to sections for appropriate time (2hrs RT) at appropriate concentration and temperature. Also applied negative control sera (normal sera from same species as primary antibody) to control sections and if possible used reagent control antibody which was well demonstrated in the tissue under investigation.
25. Washed three times with tris buffer for three minutes each.
26. Covered all sections in link reagent (Dako REAL K5355 or K5005), for 20mins RT.
27. Washed three times with tris buffer for three minutes each change.
28. Incubated in REAL streptavidin (also known as AP enzyme enhancer) (Dako K5355) with ALK phos conjugate twenty minutes at RT.
29. Washed three times with tris buffer for three minutes each change.
30. Made up Chromogen by adding 20 microlitres of chromogen to each ml of substrate and then incubated sections for ten minutes.
31. Washed in running tap water 2 minutes.
32. Rinsed in distilled water.
33. Dehydrated on hotplate.
34. Cleared in three changes of xylene (2 minutes each change) and mounted sections in Depex (or similar).
35. Then finally coverslipped using machine with 54 mm coverslips.
36. Dried on hotplate overnight.

3.5.9 CD11c immunostaining procedure

1. Paraffin blocks were sectioned at 3 microns after cooling in a -20 freezer or on ice blocks for 5-10 minutes.
2. Two sections separated by 40-50 microns (approximately 10 sections) were placed on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade).
3. The slides were dried for 2 hours in slide drying oven.
4. Sections were dewaxed in "xylene" 2 x 5 mins each in fume hood.
5. Then hydrated to water using 100% ethanol followed by 95% then 70%, 3 minutes each change.
6. Sections were rinsed well in running tap water (2 minutes).
7. Placed in 3% H₂O₂ in distilled water for 15 minutes.
8. Washed in running tap water 2 minutes.
9. Slides were placed in pressure cooker¹, for 6 minutes on high then 6 minutes at about 6.5 on dial till just expelling steam using: 500ml of citrate buffer pH6 solution with 500mls of distilled water in pressure cooker.
10. Allowed to cool to below 35 degrees C before washing in running tap water 2 minutes.
11. Washed sections well using PBS pH 7.4.
12. Applied CD11c Abcam ab52632 clone ep1347Y for 2 hours mins at room temp (dilute in dako diluent) and rabbit IgG1 at 1/20,000 for two hours at RT on serial sections.
13. Washed sections well using PBS pH 7.4 3x 3mins each change
14. Applied Dako Envision + (anti rabbit) (Dako K4003) reagent to sections for 30 mins.
15. Washed sections well using PBS 3 x changes 5 mins each change
16. Made up Invitrogen DAB plus solution using 20 micro litres of DAB to 1 ml of substrate buffer.
17. Apply DAB to sections for 10 mins.
18. Rinsed sections in running water
19. Placed sections in mayers haematoxylin to elaborate nuclei, 2 mins.

20. Rinsed in running water.
21. Placed in approx 400ml of water with 6-8 drops of ammonia 30 secs.
22. Rinsed well in running water and check nuclei (adjusted further if necessary).
23. Washed in running tap water 2 minutes.
24. Rinsed in distilled water.
25. Dehydrated in 95% then 2 x 100% ethanol for 1 minute each change.
26. Cleared in three changes of xylene (2 minutes each change) and mounted sections in Depex (or similar).
27. Then finally coverslipped using machine with 54 mm coverslips.
28. Dried on hotplate overnight.

3.5.10 CD4/CD8 immunostaining procedure

1. Paraffin blocks were sectioned at 3 microns after cooling in a -20 freezer or on ice blocks for 5-10 minutes.
2. Two sections separated by 40-50 microns (approximately 10 sections) were placed on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade).
3. Dried overnight at 37 degrees.
4. Dried on hotplate for a further hour at 56 degrees just prior to sectioning.
5. Dewaxed sections in "xylene" 2 x 5 mins each in fume hood.
6. Hydrated to water using 100% ethanol followed by 95% then 70%, 3 mins each change.
7. Rinsed sections well in running tap water (2 mins).
8. Placed in 0.5% H₂O₂ in water for 15 minutes. (5 ml of 30% H₂O₂ in 295ml of Water).
9. This was an important step since the antigens are destroyed if harsh peroxidase treatment was used. Use only 0.5% in water.
10. Washed in running water 2 minutes.
11. Added 500ml 1mM EDTA pH 8.00 in round plastic medium staining jar and placed in plastic autoclave container that has 250ml of distilled water in it (0.186g of EDTA in 400ml of distilled water, adjust pH to 8 with 1 molar sodium hydroxide and then make up to 500ml with distilled water).
12. Heated solutions without slides in microwave in autoclave (with lid in open position but rubber bung in place) for 10minutes on high.
13. Heated for 3 minutes on high in microwave with autoclave in closed position and yellow button pressed down.
14. Removed autoclave from microwave and cool rapidly in running water until yellow button drops (5 mins, care was required here, gloves or cloth was used to remove autoclave and do not open until temperature returns to RT).
15. Removed plastic staining jar from autoclave and further cool sections in water bath until temperature reached about 35 degrees C.
16. Placed slides in PBS pH 7.4, 2 minutes.

17. Applied primary antibody to sections for 2hr RT diluted to appropriate dilution in Dako antibody Diluent. (1:15) Also applied negative control sera (normal sera from same species as primary antibody) to control sections and if possible used reagent control antibody that is well demonstrated in the tissue under investigation.
18. Washed sections well using PBS 3 x changes 5 mins each change.
19. Applied Dako Envision + (Dako 003355) reagent to sections for 30 mins.
20. Washed sections well using PBS 3 x changes 5 mins each change.
21. Made up Dako DAB plus solution (K3468) using 20 micro litres of DAB to 1 ml of substrate buffer.
22. Applied DAB to sections for 10 mins.
23. Washed sections well using PBS 5 mins.
24. Rinsed sections in running water.
25. Placed sections in Mayers haematoxylin to elaborate nuclei, 1 min (or in Harris's 2 mins then differentiate with three dips in 1% acid alcohol).
26. Rinsed in running water.
27. Placed in approx 400ml of water with 6-8 drops of ammonia 30 secs.
28. Rinsed well in running water.
29. Dehydrated in 95% ethanol, then twice in 100% ethanol (2 minutes each change)
30. Cleared in three changes of xylene (2 minutes each change) and mounted sections in Depex (or similar).
31. Dried on hotplate overnight.

3.5.11 Neutrophil elastase immunostaining procedure

1. Paraffin blocks were sectioned at 3 microns after cooling in a -20 freezer or on ice blocks for 5-10 minutes.
2. Two sections separated by 40-50 microns (approximately 10 sections) were placed on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade).
3. The slides were dried for 2 hours in slide drying oven.
4. Sections were dewaxed in "xylene" 2 x 5 mins each in fume hood.
5. Then hydrated to water using 100% ethanol followed by 95% then 70%, 3 minutes each change.
6. Rinsed sections well in running tap water (2 mins).
7. Placed in 3% H₂O₂ in distilled water for 15 minutes.
8. Washed in running water 2 minutes.
9. Rinsed in working phosphate buffered saline (diluted from Tubbs stock buffer 1 in 25) 2 mins.
10. Applied Neutrophil elastase (Dako M 0752) 1/500 1hr RT (**no protease**). Also applied negative control sera (normal sera from same species as primary antibody) to control section at similar concentration.
11. Washed sections well using PBS 3 x changes 5 mins each change.
12. Applied Dako Envision + (Dako k4001) reagent to sections for 30 mins.
13. Washed sections well using PBS 3 x changes 5 mins each change.
14. Made up Dako DAB plus solution (K3468) using 20 micro litres of DAB to 1 ml of substrate buffer.
15. Applied DAB to sections for 10 mins.
16. Washed sections well using PBS 5 mins.
17. Rinsed sections in running water.
18. Placed sections in Mayers haematoxylin to elaborate nuclei, 2 mins (or in Harris's 2 mins then differentiate with three dips in 1% acid alcohol).
19. Rinsed in running water.
20. Placed in approx 400ml of water with 6-8 drops of ammonia 30 secs.
21. Rinsed well in running water.

22. Dehydrated in 95% ethanol then changed twice with 100% ethanol (2 minutes each change).
23. Cleared in three changes of xylene (2 minutes each change) and mounted sections in Depex (or similar).
24. Dried on hotplate overnight.

3.5.12 CD19 immunostaining procedure

1. Paraffin blocks were sectioned at 3 microns after cooling in a -20 freezer or on ice blocks for 5-10 minutes.
2. Two sections separated by 40-50 microns (approximately 10 sections) were placed on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade).
3. The slides were dried for 2 hours in slide drying oven.
4. Sections were dewaxed in "xylene" 2 x 5 mins each in fume hood.
5. Then hydrated to water using 100% ethanol followed by 95% then 70%, 3 minutes each change.
6. Rinsed sections well in running tap water (2 mins).
7. Placed in 3% H₂O₂ in distilled water for 15 minutes.
8. Washed in running water 2 minutes.
9. Rinsed in working phosphate buffered saline (diluted from Tubbs stock buffer 1 in 25) 2 mins.
10. Applied primary antibody to sections for 2 hours at 1/8000 dilution at room temperature. Also applied negative control sera (normal mouse sera at 1/5000) to control section and if possible used reagent control antibody which was well demonstrated in the tissue under investigation.
11. Washed sections well using PBS 3 x changes 5 mins each change.
12. Applied Dako Envision + (Dako k4001 or k4003) reagent to sections for 30 minutes.
13. Washed sections well using PBS 3 x changes 5 minutes each change.
14. Made up Invitrogen DAB solution (cat no 882014) using 20 micro litres of DAB to 1 ml of substrate buffer.
15. Applied DAB to sections for 10 minutes.
16. Washed sections well using PBS 5 minutes.
17. Rinsed sections in running water.
18. Placed sections in Mayers haematoxylin to elaborate nuclei, 2 minutes
19. Rinsed in running water.
20. Placed in approx 400ml of water with 6-8 drops of ammonia 30 seconds.

21. Rinsed well in running water.
22. Dehydrated in 95% ethanol, then two changes of 100% ethanol (2 minutes each change).
23. Cleared in three changes of xylene (2 minutes each change) and mounted sections in Depex (or similar).
24. Dried on hotplate overnight.

3.5.13 CD68 immunostaining procedure

1. Paraffin blocks were sectioned at 3 microns after cooling in a -20 freezer or on ice blocks for 5-10 minutes.
2. Two sections separated by 40-50 microns (approximately 10 sections) were placed on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade).
3. Dried on hotplate at 56 degrees one hour and overnight at 37 degrees.
4. Dewaxed sections in "xylene" 2 x 5 mins each in fume hood.
5. Hydrated to water using 100% ethanol followed by 95% then 70%, 3 mins each change.
6. Rinsed sections well in running tap water (2 mins).
7. Placed in 3% H₂O₂ in distilled water for 15 minutes.
8. Washed in running water 2 minutes.
9. Rinsed in working phosphate buffered saline (diluted from Tubbs stock buffer 1 in 25) 2 mins.
10. Pre-incubated them in PBS at 37 degrees in water bath for 5 mins.
11. Incubated in 0.025% protease type VIII (Sigma P5380) in PBS for three minutes at 37 degrees in water bath.
12. Washed in running tap water 5 minutes.
13. Covered slides with PBS pH 7.4.
14. Applied primary antibody to sections for 30 mins at room temp diluted to 1/500 in Dako antibody diluent. Also applied negative control sera (normal sera from same species as primary antibody) to control sections and if possible used reagent control antibody which was well demonstrated in the tissue under investigation.
15. Washed sections well using PBS 3 x changes 5 mins each change.
16. Applied DAKO Envision + (Dako k4001) reagent to sections for 30 mins.
17. Washed sections well using PBS 3 x changes 5 mins each change.
18. Made up Dako DAB plus solution (K3468) using 20 micro litres of DAB to 1 ml of substrate buffer.
19. Applied DAB to sections for 10 mins.
20. Washed sections well using PBS 5 mins.

21. Rinsed sections in running water.
22. Placed sections in Mayers haematoxylin to elaborate nuclei, 2 mins (or in Harris's 2 mins then differentiate with three dips in 1% acid alcohol).
23. Rinsed in running water.
24. Placed in approx 400ml of water with 6-8 drops of ammonia 30 secs.
25. Rinsed well in running water.
26. Dehydrated in 95% ethanol then changed twice in 100% ethanol (2 minutes each change).
27. Cleared in three changes of xylene (2 minutes each change) and mount sections in Depex (or similar).
28. Dried on hotplate overnight.

3.5.14 General procedure for Haematoxylin and Eosin (H&E) stain

1. Paraffin blocks were sectioned at 5 microns and were picked on APTS coated slides.
2. Dried overnight at 37 degrees or on hotplate at 56 degrees two hours.
3. Dewaxed sections in fresh “xylene substitute (Shandon- kept in flame cupboard)” 2 x 5 mins each in fume hood.
4. Hydrated to water using 100% ethanol followed by 95% then 70%, 2 mins each change.
5. Rinsed sections well in running tap water (2 minutes).
6. Placed sections in Mayer’s haematoxylin to elaborate nuclei, 5 minutes.
7. Rinsed in running water.
8. Placed in approx 400ml of water with 8 drops of ammonia 30 seconds.
9. Rinsed well in running water.
10. Checked nuclei, if they are sufficiently stained using microscope.
11. Placed in eosin solution 2 minutes.
12. Rinsed quickly in running water to remove excess eosin and place into 95% ethanol 30 seconds with agitation.
13. Further dehydrated with 3 changes of 100% ethanol 1 min each change.
14. Cleared sections using 3 changes of fresh substitute xylene 2 minutes each change.
15. Placed sections in Xylene for 1 min prior to coverslipping with xylene based mountant (Depex or similar).
16. Dried on hotplate overnight.

Chapter 4

Rbm fragmentation and potential EMT in the airways of smokers is exaggerated in COPD

4.1 Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by progressive fixed airflow limitation and associated chronic airway inflammation; usually due to tobacco smoke inhalation (Barnes, 2003; Barnes et al., 2003). One of the features of chronic airway diseases, including COPD, is airway remodelling (Churg et al., 2006; J. C. Hogg et al., 1968; Nakano et al., 2002; Sassetta et al., 2000). In COPD the mechanism and structural changes of remodelling are poorly described.

A new explanation for remodelling in inflammatory respiratory diseases, which may be applicable to COPD, is transition of airway epithelial cells to a mesenchymal phenotype and then migration through the Rbm to the sub epithelial lamina propria, a process termed epithelial mesenchymal transition (EMT) (Peter K. Jeffery, 2004; Brigham C. Willis et al., 2006). However, to our knowledge, there have been no reported studies undertaken examining markers of EMT in human airway biopsy tissues from subjects with COPD, although EMT was recently demonstrated in the similar bronchiolitis obliterans syndrome (BOS) that occurs as a manifestation of chronic rejection post lung transplant. (Ward et al., 2005)

In preliminary observations (Chapter 1) I found that the Rbm in smokers was significantly fragmented, which can be a hallmark of EMT (Acloque et al., 2009; R. Kalluri, 2009; Raghu Kalluri & Neilson, 2003; R. Kalluri & Weinberg, 2009; Zeisberg & Neilson, 2009). This encouraged me to test the hypothesis that the airway epithelium is activated in smokers, promoting EMT, but that this will be especially active in COPD. As the first such study that I am aware of in COPD, I examined

airway biopsies for expression of markers generally accepted to represent EMT proteome, including matrix metalloproteinase-9 (MMP-9), which can digest type IV collagen, (Ward et al., 2005) S100A4 (also known as fibroblast specific protein 1, Fsp1) and epidermal growth factor receptor (EGFR) as a marker of epithelial activation, and vimentin to further strengthen the possibility of EMT (R. Kalluri & Weinberg, 2009).

I have investigated current smokers with established COPD (CS) and also current smokers with normal lung function (NS), comparing them to ex-smokers with COPD (ES) and normal healthy, never-smoking controls (NC) using airway biopsies. This is a detailed study of Rbm fragmentation and a preliminary study of EMT in COPD, and provides novel data and potentially important new insights into the pathogenesis of airway disease in smokers and COPD.

4.2 Methods and materials

4.2.1 Subjects

The study was approved by institutional Ethics Committees (details given in Chapter 3). Subjects were recruited by advertisement. All subjects signed informed consent. Subjects with a history of other respiratory disorders were excluded. The diagnosis of COPD was made according to GOLD guidelines (GOLD, 2007). None were receiving corticosteroids (oral or inhaled).

4.2.2 Bronchoscopy

Details of bronchoscopic procedures are given in Chapter 3.

4.2.3 Immunostaining

Sections were stained with these monoclonal antibodies: anti-MMP-9, anti-EGFR and anti-S100A4 polyclonal together with a horseradish peroxidase (HRP) conjugated DAKO Envision plus reagent for secondary antibody binding and colour resolution using diaminobenzidine (DAB). Double staining with vimentin was elaborated using an anti-vimentin monoclonal antibody and then secondarily bound

using Dakocytomation “Real” alkaline phosphatase reagents and visualised using Dakocytomation liquid permanent red (details given Chapter 3).

4.2.4 Biopsy analysis

Computer-assisted image analysis was performed with a Leica DM 2500 microscope (Leica Microsystems, Germany), Spot insight 12 digital camera and Image Pro V5.1 (Media Cybernetics, USA) software. Cellular and other changes, e.g. in the Rbm, were normalised for purposes of comparisons in terms of the total length of basement membrane assessed. EGFR was measured as percentage of epithelium stained for EGFR over total basement membrane length (details given in Chapter 3).

4.2.5 Statistical analysis

Results are presented as medians and ranges. A non-parametric ANOVA was undertaken, and specific group differences then explored using the Mann Whitney U test. Associations between variables were assessed using Spearman's rank test. Statistical analyses were performed using SPSS 15.0 for Windows, 2003, with a two-tailed P -value ≤ 0.05 being considered statistically significant. Linear regression analysis for potential confounders was undertaken, and differences between groups in gender balance, age, and atopy were found to be non-contributory.

4.3 Results

The group demographics of subjects who participated in the study are presented in the table given below:

Table 4: Demographic and lung function data for subjects.

Groups (numbers)	COPD-CS (n=17)	COPD-ES (n=15)	NS (n=16)	NC (n=15)
GOLD I/GOLD II [‡]	10/7	8/7	N/A	N/A
Male/female	9/8	9/6	12/4	7/8
Age (years)	61 (46-78) (p=0.001) [†]	62 (53-69) (p=0.001) [†]	50 (30-66) (p=0.31)	44 (20-68)
Smoking (pack years)	45 (18-78)	51 (18-150)	32 (10-57)	0
FEV1% predicted (Post BD) [★]	83 (66-102) (p<0.001) [†]	83 (54-104) (p<0.001) [†]	99 (78-125) (p=0.01) [†]	113 (86-140)
FEV1/FVC % (Post BD) [★]	59 (46-68) (p<0.001) [†]	57 (38-68) (p<0.001) [†]	77 (70-96) (p=0.22)	82 (71-88)

Data expressed as median and range

[†]Significance difference from NC

[★] Post BD values after 400µg of salbutamol

[‡] Diagnosis of COPD was made according to GOLD guidelines (GOLD, 2007)

4.3.1 Fragmentation and presence of “clefts” within the Rbm

Following our preliminary observation of linear “clefts” or elongated spaces/cracks in the Rbm, we have pragmatically used this for quantifying the degree of fragmentation. However, there were also commonly fragments of Rbm “hanging off” and similar pieces seemingly separated from it, albeit in the largely two-dimensional sections (Figure 4.1). This fragmentation was very rarely present in normal controls. The length of the individual “clefts” was measured, summed and results expressed as percentage of the total Rbm length to give a comparable measure of the degree of Rbm fragmentation for each individual. The number of clefts was significantly ($p<0.001$) higher in CS, NS and ES compared to NC (Figure 4.2 and 4.3). The finding was more pronounced in current smoking COPD subjects. There was a significant positive correlation ($R=0.41$, $p=0.02$) between smoking (pack years) and percentage of clefts in Rbm in both COPD current smokers and ex-smokers (Figure 4.4) but not in NS. However, no association was found with lung function measurements.

4.3.2 MMP 9 positive cells associated with Rbm

There were significantly more cells that stained positively for MMP-9 (Figure 4.2) in the Rbm per mm in CS, NS and ES compared to NC ($p<0.002$) (Figure 4.5). These cells were located predominantly within the “clefts”, but were also present immediately below and just adjacent to the Rbm (Figure 4.2) and were included on a single count. There was some minor degree of MMP-9 staining of cells within the airway epithelium, counted separately, with no difference between the groups. No association was found with lung function measurements.

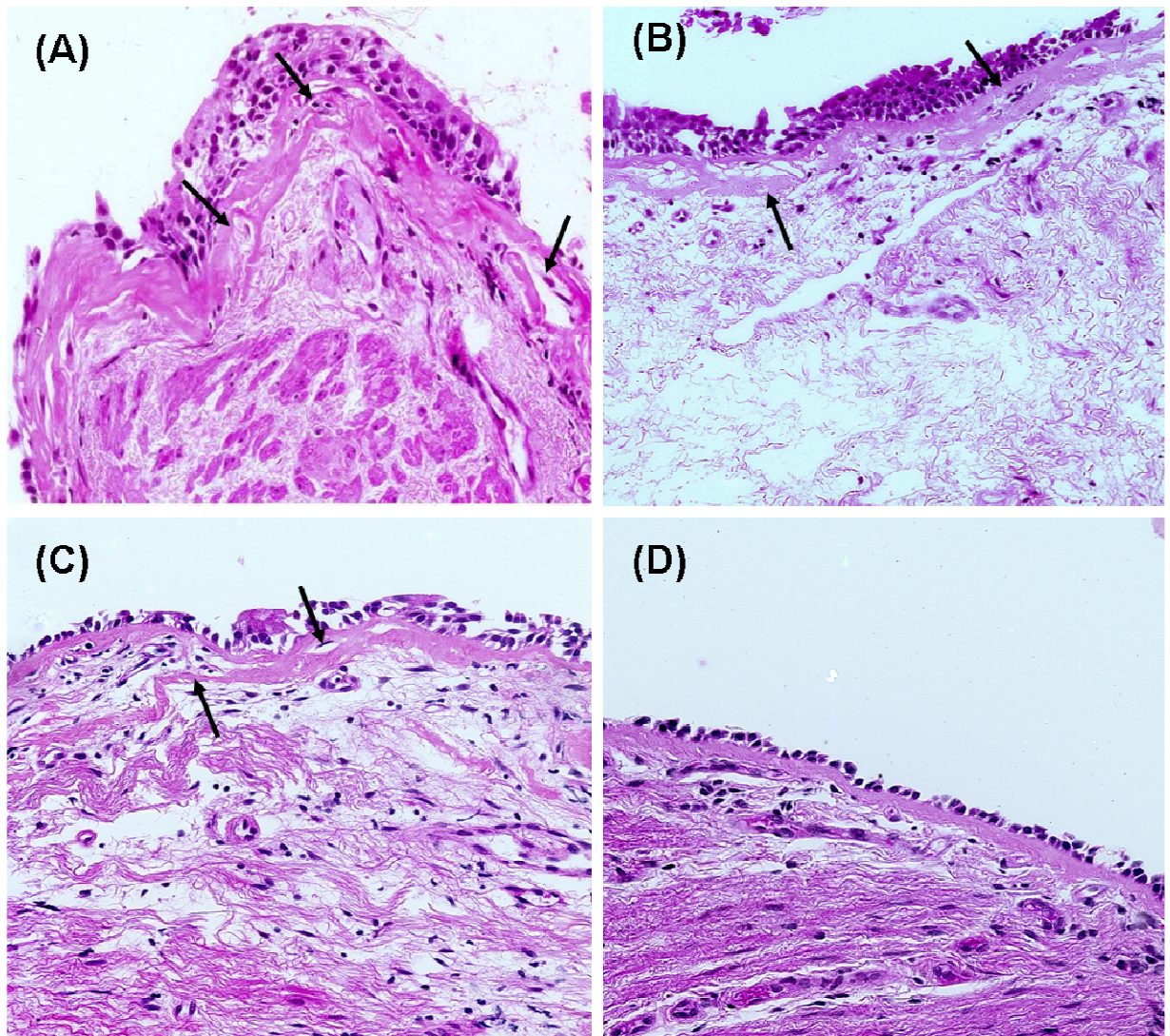


Figure 4.1 : Bronchial biopsy section from (A) COPD current smoker; (B) COPD ex-smokers; (C) normal lung function smoker; compared to a (D) normal control: black arrows indicating Rbm fragmentation with many “clefts” evident with cells within them. Original magnifications, x 400. Stain: haematoxylin and eosin (H & E).

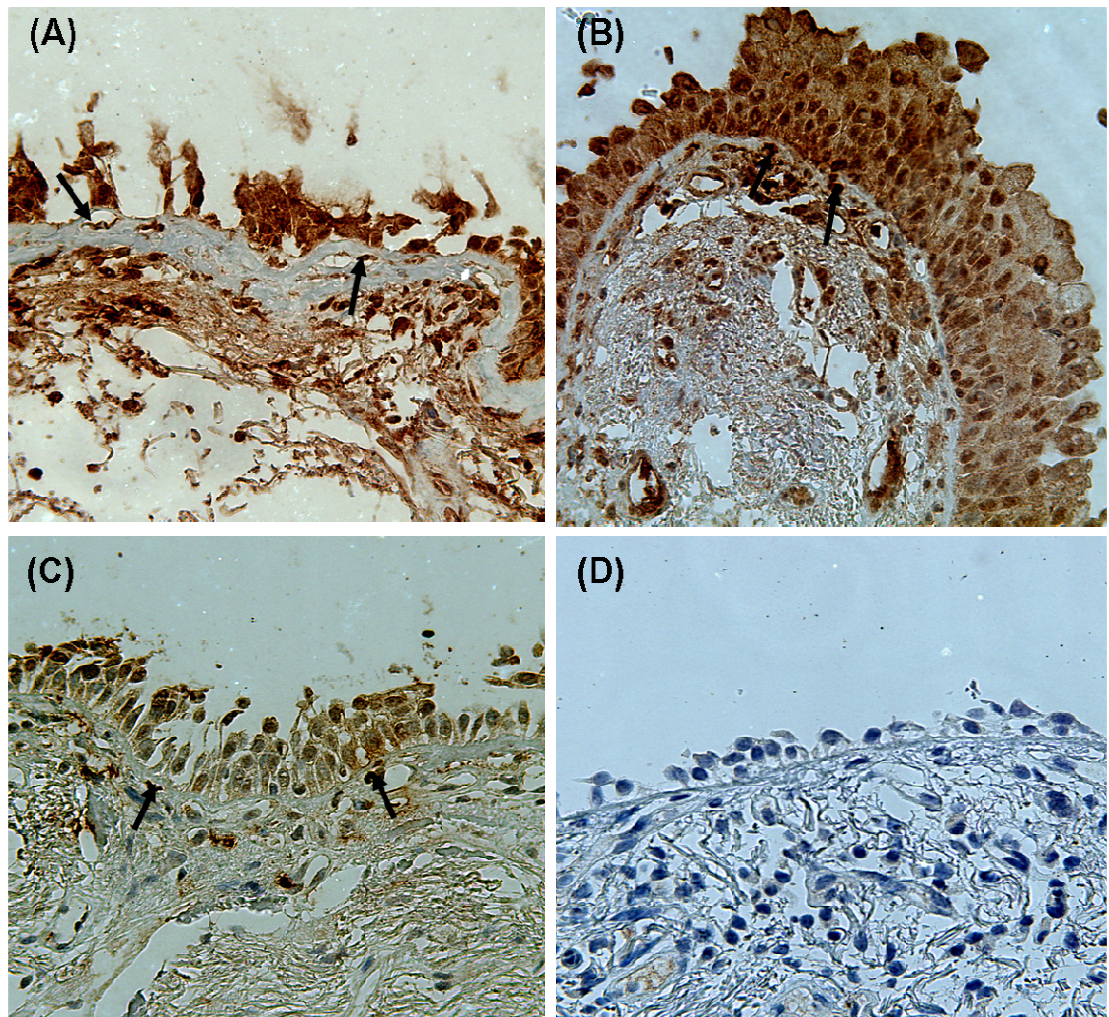


Figure 4.2: Immunohistochemical staining of bronchial biopsy sections for MMP-9 from: (A) COPD-CS (B) COPD-ES (C) NS compared to (D) NC, with black arrows indicating clefts with cells positive for MMP-9. Original magnifications, x 630.

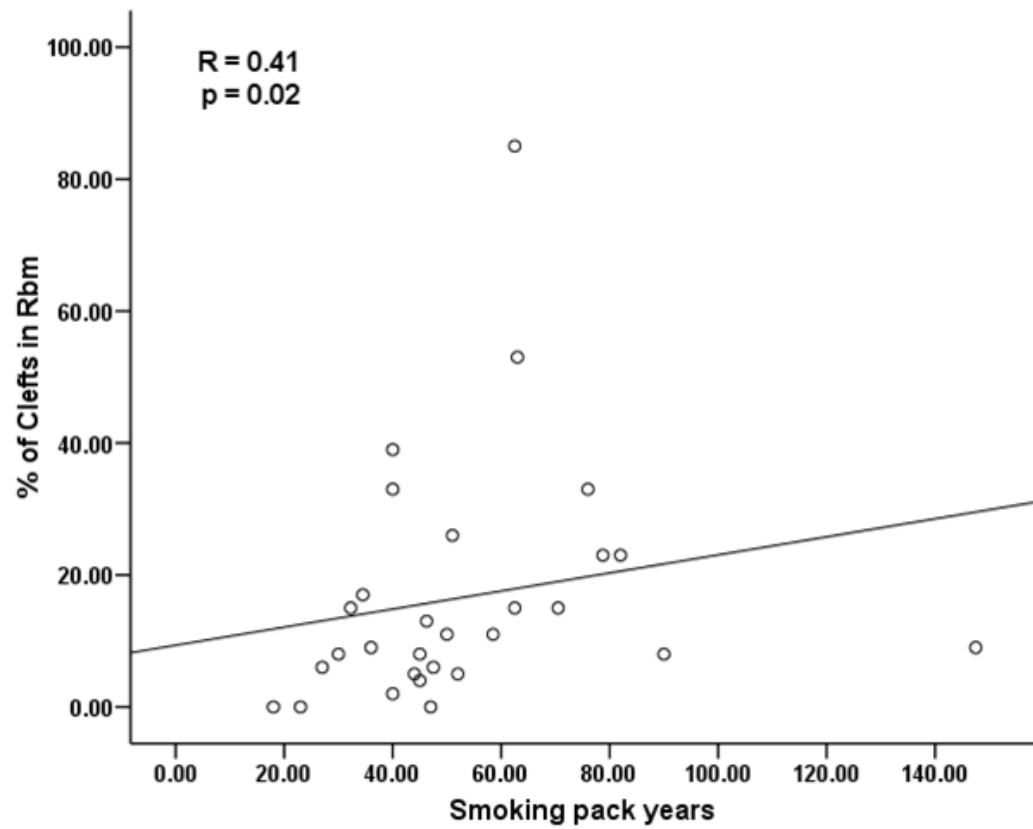


Figure 4.4: Correlation between **percentage of clefts in Rbm length examined** and smoking (pack years) in COPD current smokers and ex-smokers.

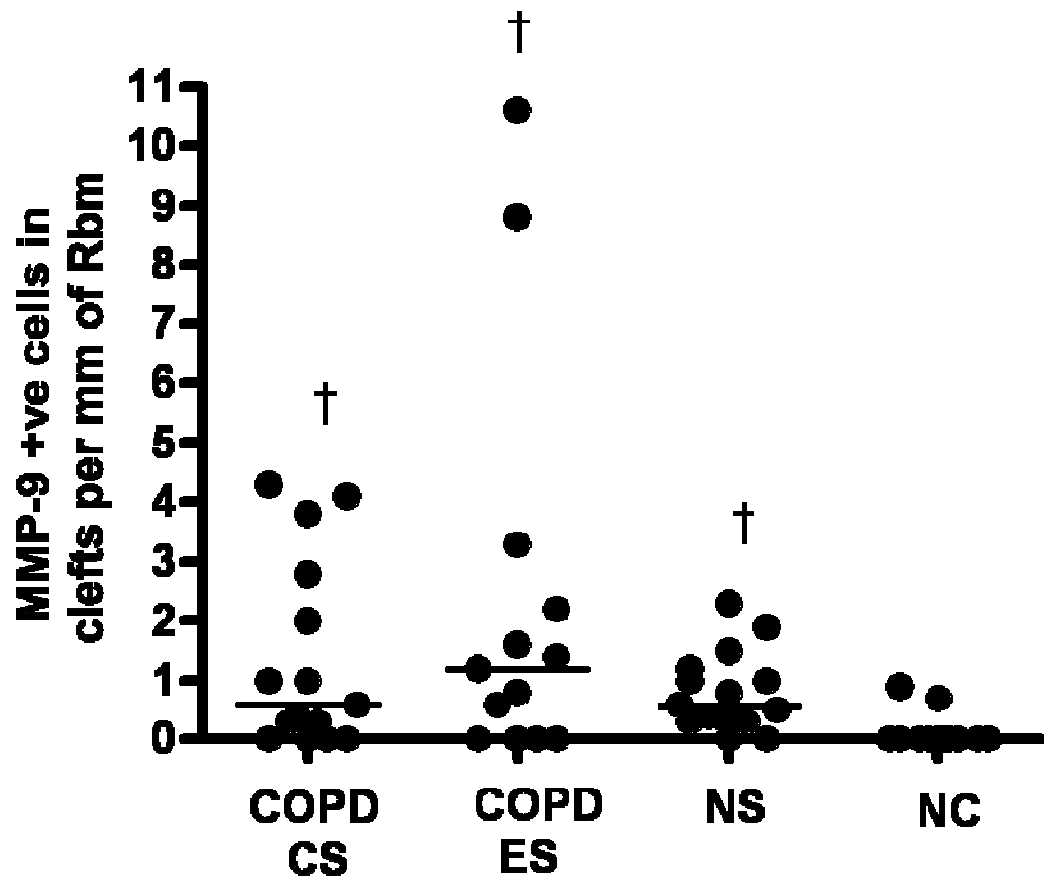


Figure 4.5: Number of MMP-9 positive cells in clefts per mm of Rbm; †significant difference from NC ($p < 0.002$).

4.3.3 S100A4 positive cells in basal epithelium

There were marked increases in cells staining for S100A4 within the basal layers (Figure 4.6) of the airway epithelium compared to NC in CS, NS and ES ($p < 0.004$; Figure 4.7). There was a significant negative correlation ($R = -0.49$, $p = 0.04$) between basal cell S100A4 staining and FEV1/FVC % in COPD current smokers (Figure 4.8), i.e. with airflow obstruction.

4.3.4 S100A4 positive cells in Rbm

The Rbm showed cells positively staining for S100A4 cells (Figure 4.6), but not exclusively within the clefts. This was significantly higher in CS, NS and ES than in NC per mm of Rbm ($p < 0.001$). In addition, there was also a significant difference between CS compared to ES and NS ($p < 0.007$) for S100A4 positive cells in Rbm per mm (Figure 4.9). No association was found with lung function measurements.

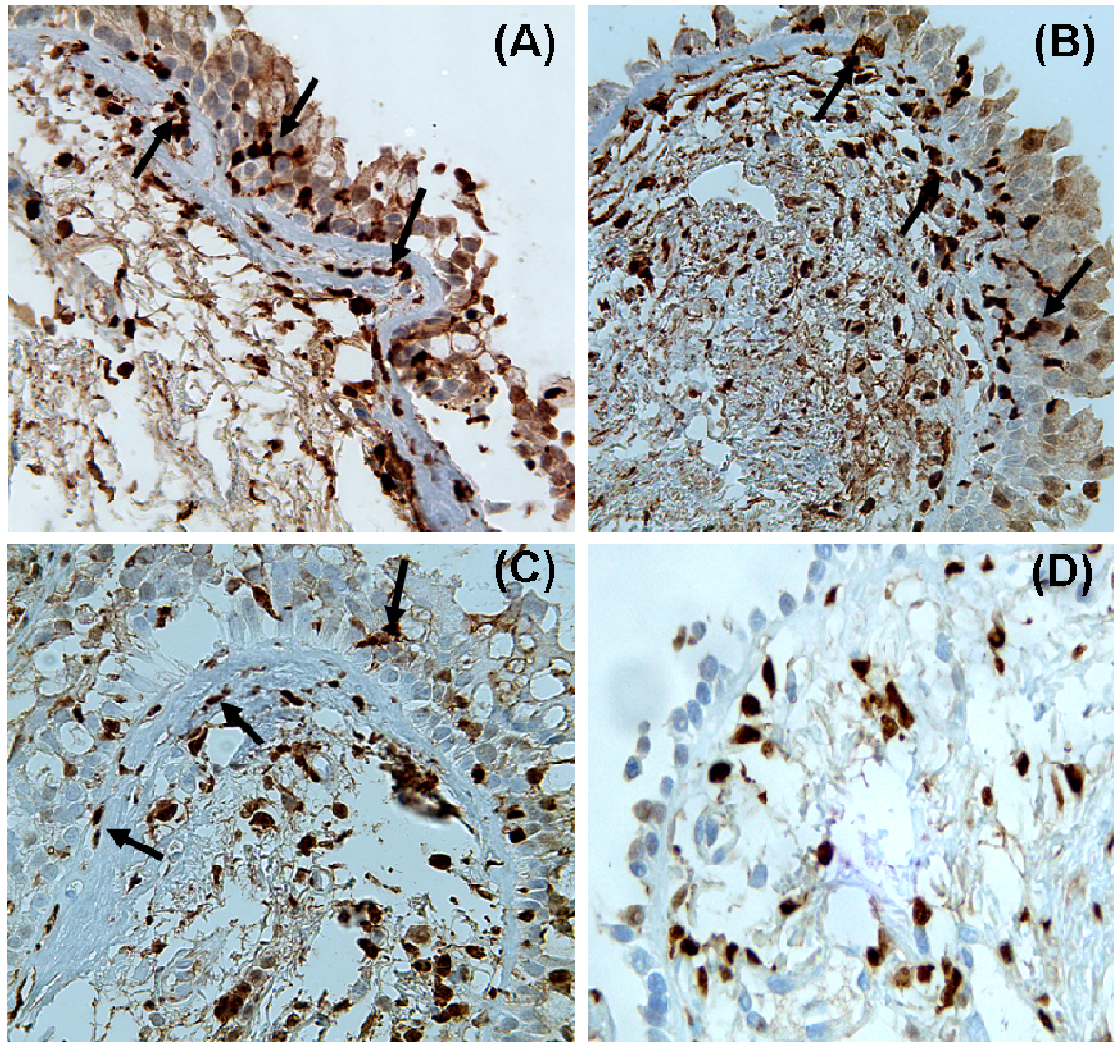


Figure 4.6: Immunohistochemical staining of bronchial biopsy sections for S100A4 from: (A) COPD-CS (B) COPD-ES (C) NS compared to (D) NC, with black arrows indicating clefts, cells positive for S100A4 in the Rbm and in the basal epithelium. Original magnifications, x 630.

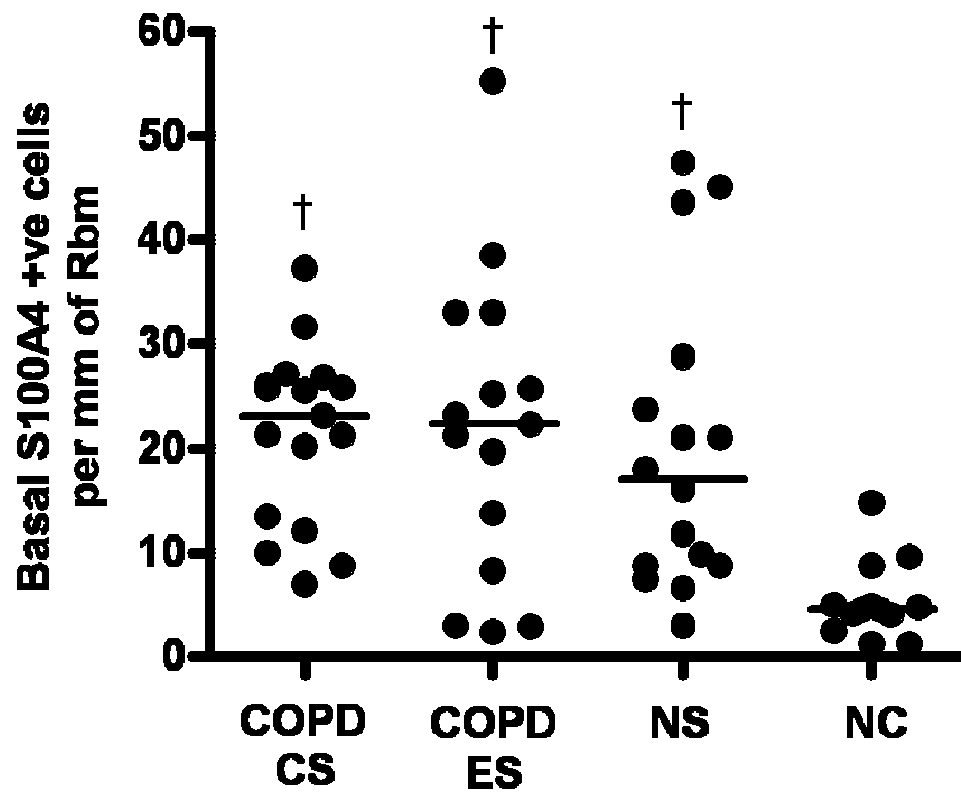


Figure 4.7: Number of basal epithelial cells positive for S100A4, per mm of Rbm; †significant difference from NC ($p < 0.004$).

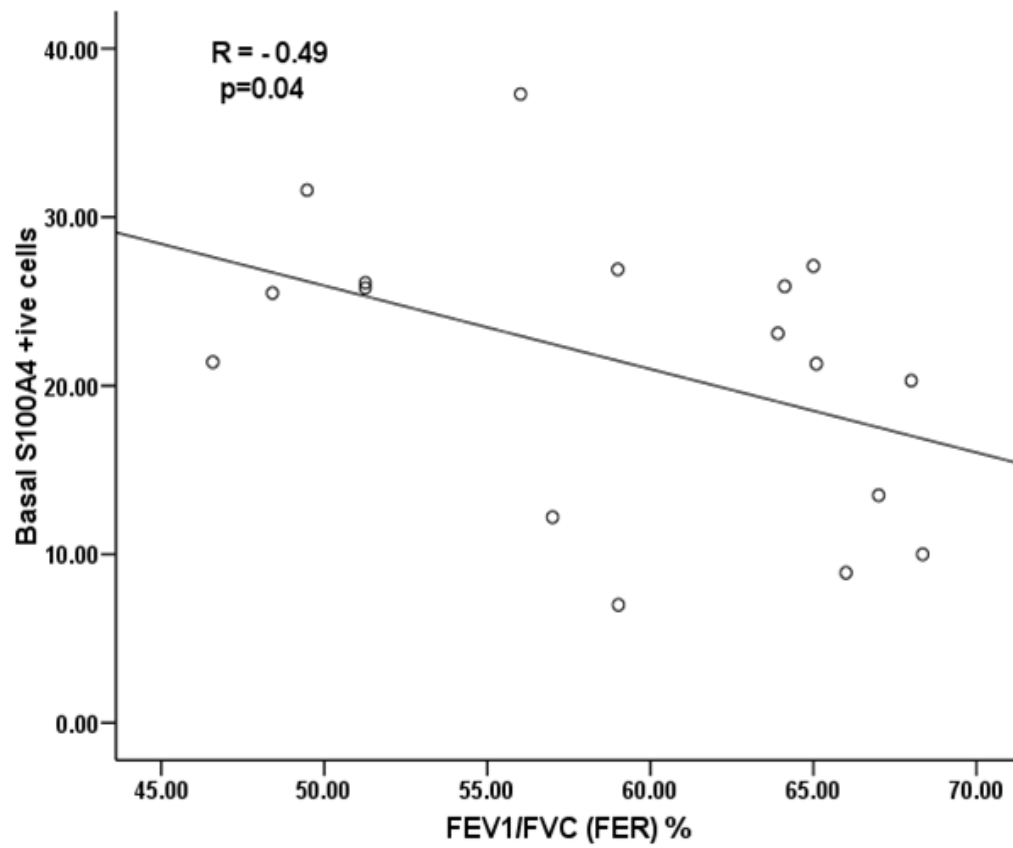


Figure 4.8: Correlation between **basal S100A4 positive cells per mm of Rbm** and **FEV1/FVC (FER) %** as index of airflow obstruction, in COPD current smokers.

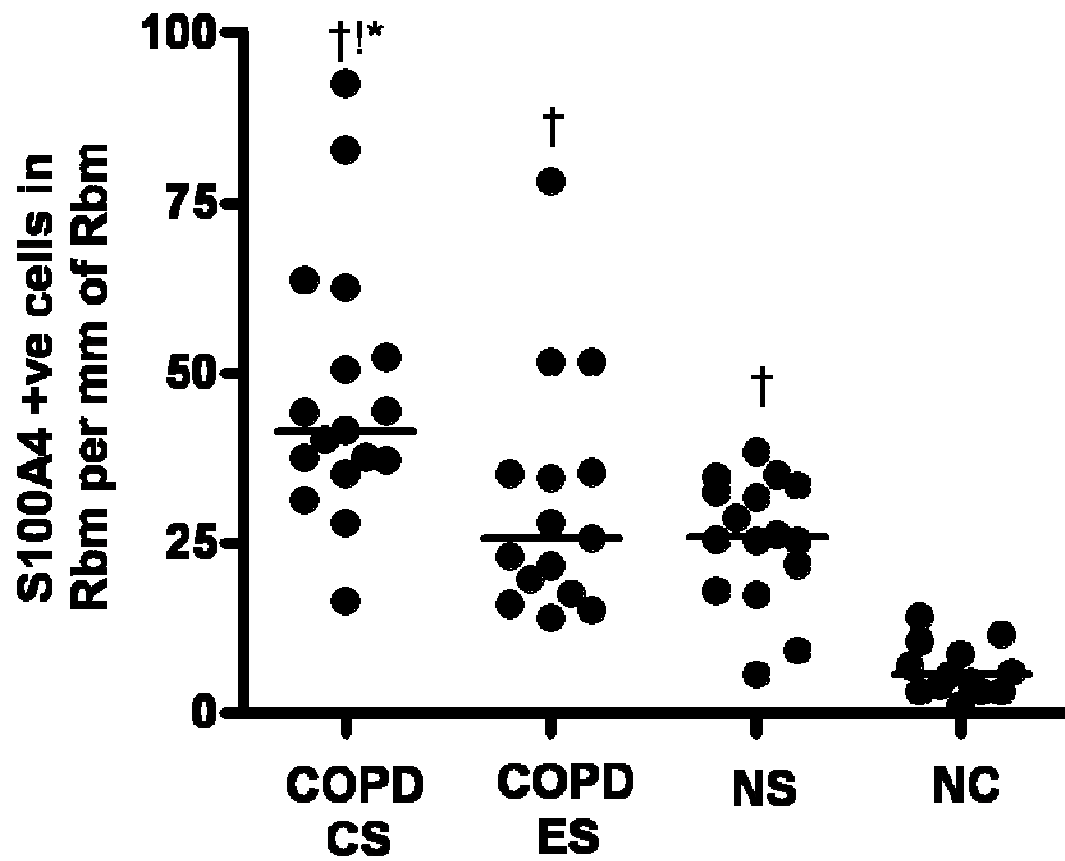


Figure 4.9: Number of S100A4 positive cells within the Rbm, per mm of Rbm; †significant difference from NC ($p < 0.001$); ! significant difference from COPD-ES ($p = 0.006$); *significant difference from NS ($p < 0.001$).

4.3.5 Double immunostaining of sections for S100A4 and vimentin

To further strengthen the evidence for EMT, we double stained and assessed multiple representative sections for COPD-CS subjects for S100A4 and the mesenchymal marker vimentin simultaneously from the same tissue block (Figure 4.10). In the basal epithelium nearly the same percentage of cells were stained for S100A4 (15.6 %) and vimentin (13.2 %) separately, and 13.4 % were double positive. In Rbm a very similar percentage of cells stained for S100A4 (19.6 %) and vimentin (23.0 %) separately, while 22.4 % cells were double positive.

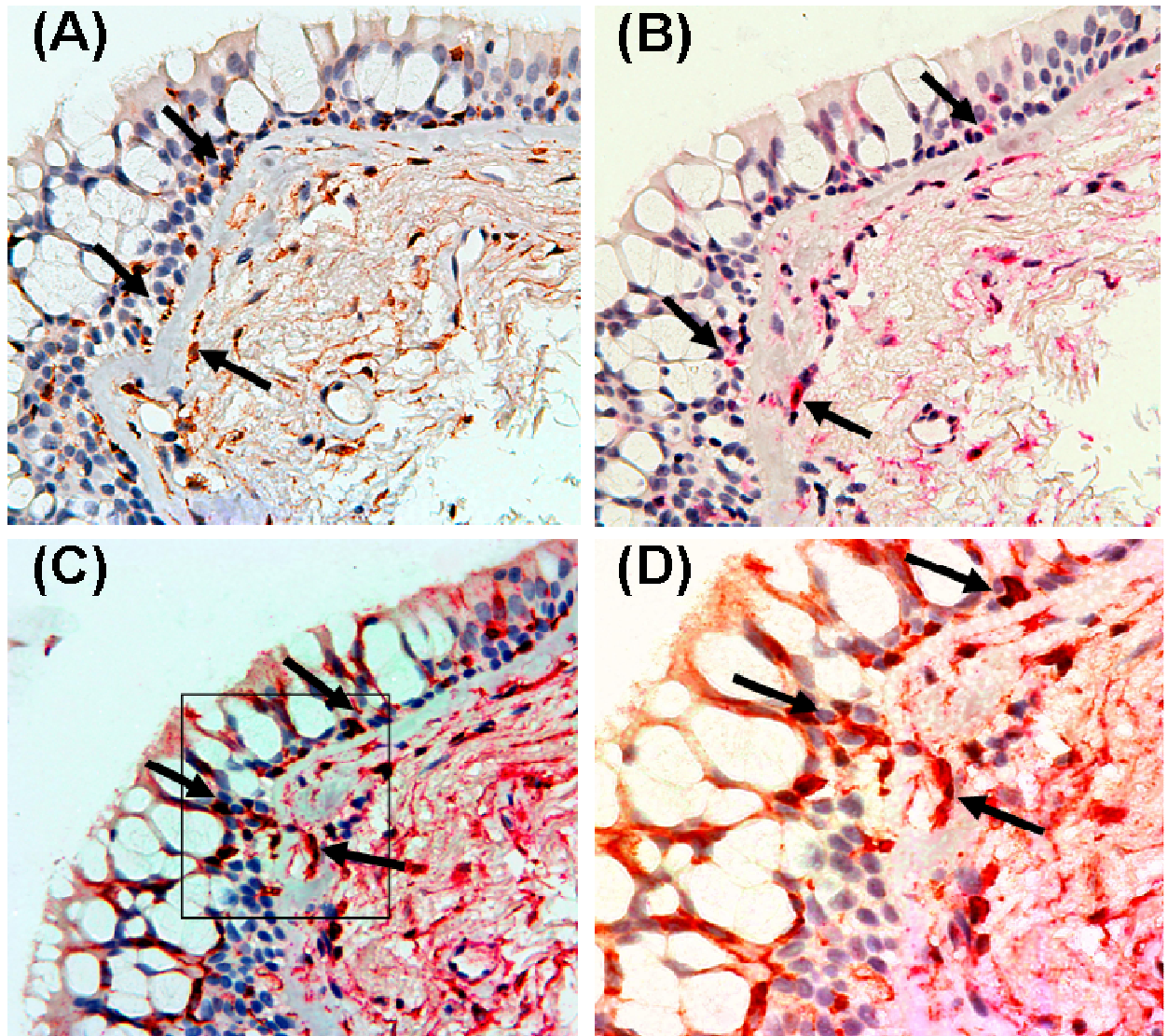


Figure 4.10: Bronchial biopsy section from a COPD current smoker, with black arrows indicating staining of cells positive for: (A) S100A4 (brown), stained with anti-S100A4 polyclonal antibody (B) vimentin (red), stained with anti-vimentin monoclonal antibody and (C) double stained for both S100A4 and vimentin (brown and red) in the basal epithelium and Rbm from the same tissue block . Original magnifications, x 630 (D) area high-magnification (100 x /1.30 oil PH 3) picture of the boxed area shown in (C).

4.3.6 EGFR expression in the airway epithelium

A greater proportion of the airway epithelium stained positive for EGFR (Figure 4.11) in the CS, NS and ES subjects compared to NC ($p < 0.001$; Figure 4.12). Once again, no association was found with lung function measurements.

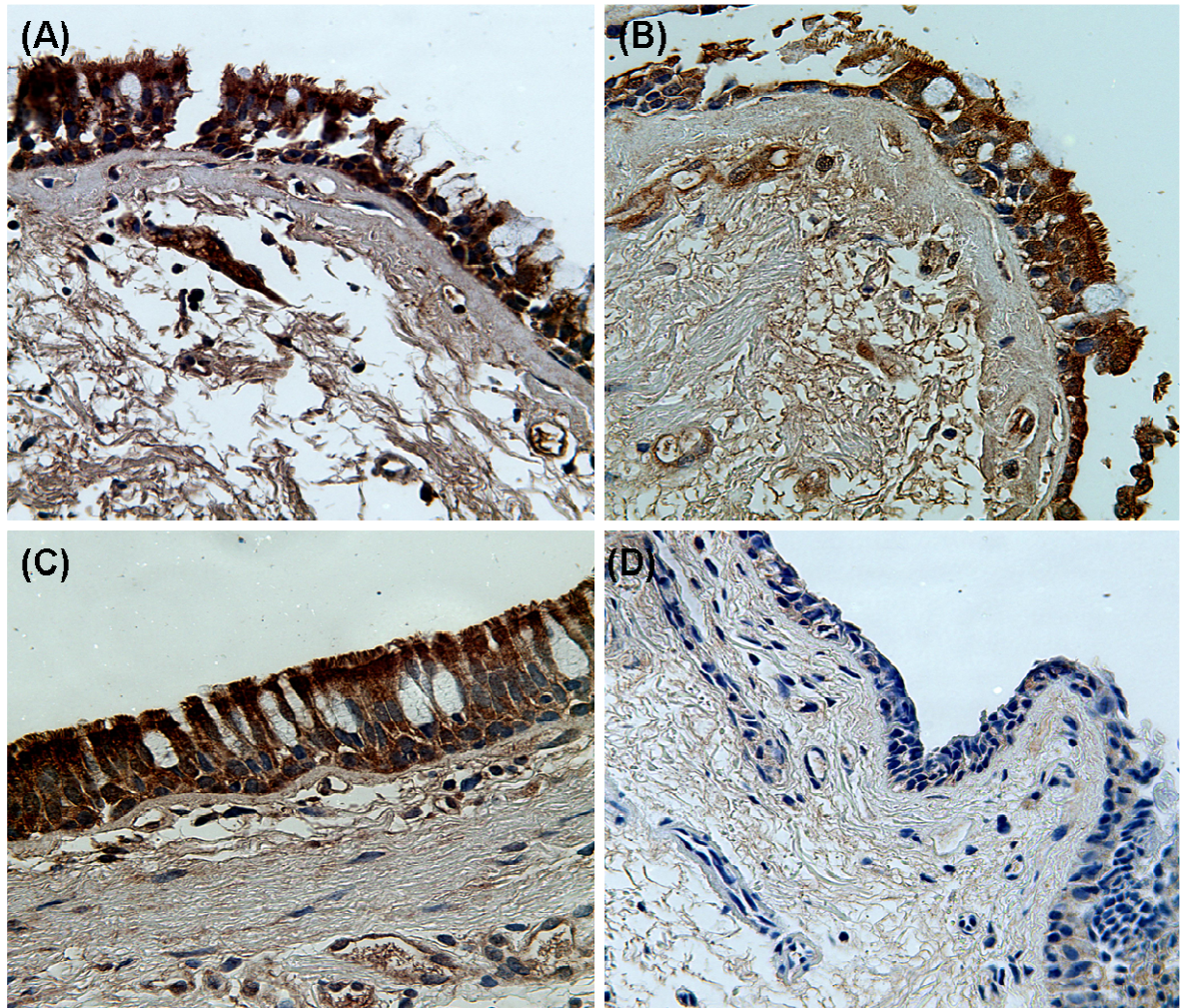


Figure 4.11: Immunohistochemical staining of bronchial biopsy sections for EGFR from: (A) COPD-CS (B) COPD-ES (C) NS compared to (D) NC, intense brown staining for EGFR in the epithelium. Original magnifications, x 630.

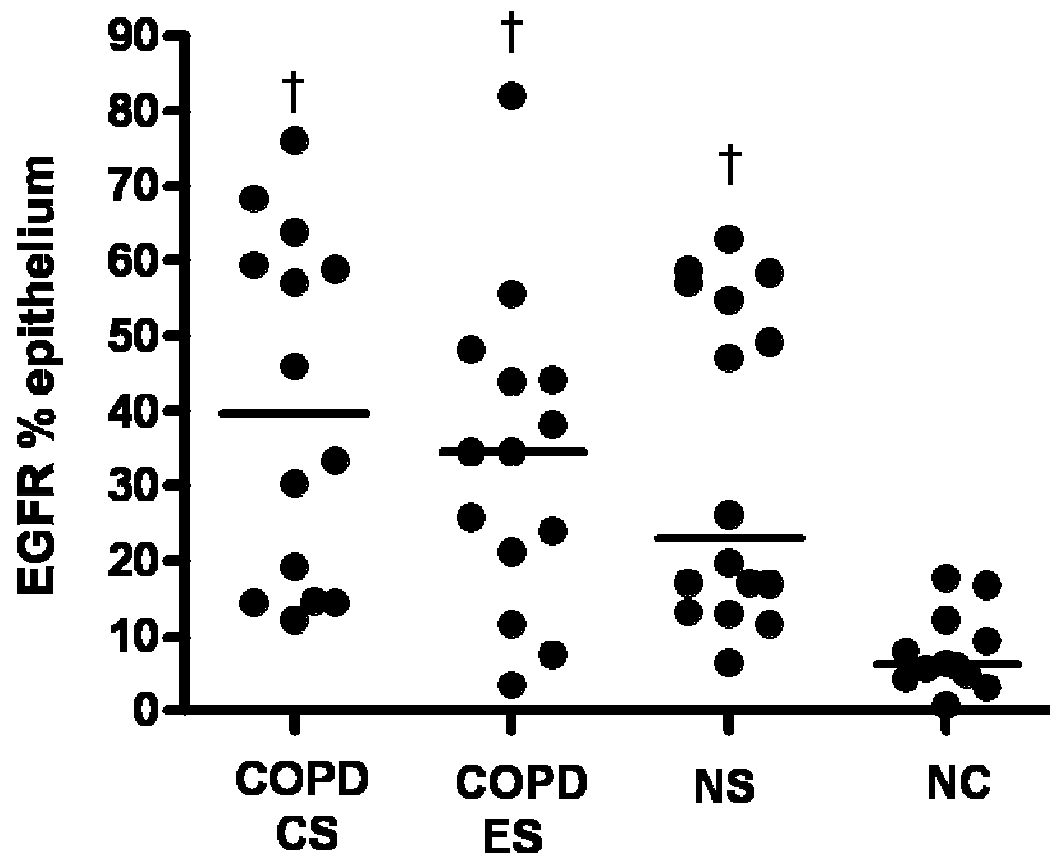


Figure 4.12: Percentage of epithelium stained for EGFR; †significant difference from NC ($p < 0.001$). Horizontal bars represent the median for each group.

4.4 Discussion

This is the first description of marked Rbm “remodelling” with fragmentation represented by quantifiable clefts, in airway wall biopsies from smokers and COPD patients, especially in current smokers. “Clefts” within the disrupted Rbm contained cells expressing the proteolytic enzyme MMP-9 and the early fibroblast-transition marker S100A4. Superficial to the Rbm, the airway epithelium stained strongly for cells expressing the activation marker EGFR. There was also marked staining with S100A4 in cells within the deeper basal layer of the airway epithelium, situated in close proximity to the Rbm, in smokers and COPD. Dual immunostaining for S100A4 and vimentin in the basal epithelium and Rbm further strengthened the likelihood that these cells are undergoing transition to a mesenchymal phenotype. We suggest that these findings indicate active migration of cells from the epithelium into and presumably through the Rbm, with these cells possessing proteolytic capacity and a fibroblast phenotype.

In general, current smokers with COPD had greater differences in these novel changes compared to normal controls than did physiologically normal smokers, although the differences overall were not statistically significant. Given the relatively small numbers of subjects, this probably reflects a Type-2 statistical error. The ex-smoker COPD group was more variable but mostly quite similar to COPD. For S100A4 cells in the Rbm, there were significant differences between current smoking COPD and both other comparator groups (COPD-ES and NS). The only significant relationship with degree of physiological fixed airflow obstruction was with S100A4 cells in the basal layer of the epithelium in the current smoking COPD group. These data taken together suggest that the remodelling changes are more exaggerated in established COPD as might be expected, especially in active current smokers, and that the changes we describe and attribute to likely EMT, may be of pathophysiological relevance.

Epithelial EGFR expression suggests that these cells are possibly primed for migration. (de Boer et al., 2006; Raghu Kalluri & Neilson, 2003) The positive findings in smokers with normal lung function, compared to normal non-smokers, suggests that smoking itself has the potential to initiate EMT. Furthermore, our data

in the ex-smoker COPD group suggest that once these changes are initiated they may be irreversible, though longitudinal smoking cessation studies will be needed to fully clarify that. However, the difference between COPD current smokers versus COPD ex-smokers for expression of S100A4 in Rbm with no difference in basal epithelial cell S100A4 staining may suggest that migration from the epithelium across the Rbm decreases following smoking cessation.

One of the common features of chronic inflammatory airway diseases, including COPD, is thought to be airway remodelling, although this has been predominantly studied in asthma. As a simile to the epithelium-mesenchymal transition suggested in BOS (Ward et al., 2005) it is likely that in COPD, the epithelium gets damaged and/or activated by irritants such as cigarette smoke constituents, and this stimulates deposition of collagen from myofibroblasts in the lamina propria. The precise origin of these primary effector cells of fibrosis in the human airway is not yet established, but there are several potential sources of fibrogenic cells within the airway wall: first, there may be expansion of a fibroblast pool by in-situ proliferation in response to injury which may lead to pathological fibrosis; (Phan, 2002) second, recruitment of circulating fibrocyte progenitors into the airway wall; (Hashimoto et al., 2004) and third, transition of airway epithelial cells via EMT (Peter K. Jeffery, 2004; Brigham C. Willis et al., 2006). Our current study provides, for the first time that we are aware of, preliminary evidence suggesting that EMT may well be active in the airway wall in smokers and COPD. EMT has only recently been recognised in the human lung/airway, (Ward et al., 2005; Brigham C. Willis et al., 2006) but it is well described in lung embryogenesis, (J. M. Lee et al., 2006) metastatic malignant disease (Bjornland et al., 1999) and as part of the repair process in renal disease following tissue injury (Yanez-Mo et al., 2003). Active EMT is indicated by the degradation of underlying basement membrane and development of a mesenchymal cell with migratory potential that moves away from the epithelium in which it originated (R. Kalluri & Weinberg, 2009; Zeisberg & Neilson, 2009).

Our finding in smokers and COPD subjects of Rbm fragmentation is quite consistent with other studies that suggest basement membrane fragmentation as a key indicator of EMT in-vivo (Acloque et al., 2009; R. Kalluri, 2009; Raghu Kalluri & Neilson, 2003; R. Kalluri & Weinberg, 2009; Zeisberg & Neilson, 2009). In retrospect, and

with our new insights, we have noted Rbm fragmentation in work published by other investigators, (for e.g. de Boer et al., 2006) but this has not previously been commented on. Within the “clefts” in the Rbm, we observed cells expressing MMP-9 and S100A4 and vimentin, and we suggest that these cells have originated from the surface airway epithelial layer. It is also possible that the cells in the clefts have migrated from the lamina propria, but against this explanation was the presence of S100A4 and vimentin staining cells in the basal layer of the epithelium in smokers and COPD, and the accompanying increase in expression of EGFR in the epithelium. These observations strongly suggest that the most likely origin of the S100A4 positive cells apparent within the Rbm is airway epithelial cells (Raghu Kalluri & Neilson, 2003).

A potential criticism of our findings is our attribution of mechanistic interpretation to “static” immunohistochemical observations and that the Rbm fragmentation presented may be an “artefact” of tissue processing. Against this is that clefts contained cells (and to some extent vessels, data not shown) and also that tissues from normal healthy controls and asthmatics (data not shown) were processed in exactly the same way, but rarely showed Rbm fragmentation. In COPD the Rbm is variably thickened, but not significantly so as in asthma (data not shown). The simultaneous occurrence of Rbm fragmentation and presence of MMP-9 and S100A4 cell staining and EGFR expression in the epithelium within the “remodelled” airways of subject with established COPD offers a biologically plausible hypothesis of cause and effect, which fits with current ideas on EMT is chronic inflammatory disease pathogenesis (Acloque et al., 2009; R. Kalluri, 2009; Raghu Kalluri & Neilson, 2003; R. Kalluri & Weinberg, 2009; Zeisberg & Neilson, 2009). Our observations indicate the potential for EMT due to smoking and especially evident in smoking COPD, but are obviously limited by the fact that they are invasive human studies. Further studies may require further EMT signals and molecular markers to confirm and extend these preliminary but novel and exciting findings.

In summary, I have demonstrated that smoking is likely to initiate EMT and resulting Rbm fragmentation and cleft formation, which is associated with cells expressing the proteolytic enzyme MMP-9. Increased epithelial expression of EGFR suggests that epithelial cells are activated and therefore primed for migration. Increased cellular

co-expression of the early EMT fibroblast-transition marker S100A4 and vimentin also suggests phenotypic transition. All of these changes seem to be especially amplified in COPD, suggesting that EMT may be initiated by smoking, and also may be a particular feature of COPD pathophysiology.

Chapter 5

Confirming EMT-Excluding potential confounding by inflammatory cell infiltrate

5.1 Introduction

In the previous Chapters I have described and detailed a comprehensive cross-sectional study looking at the potential for active EMT in COPD and I found that this was indeed very likely on the basis of “classic” published criteria. I have shown that Rbm in COPD is highly fragmented, with elongated spaces or cracks, termed “clefts”. This I believe is the primary hallmark of EMT, strongly supported in the literature (Acloque et al., 2009; R. Kalluri, 2009; Raghu Kalluri & Neilson, 2003; R. Kalluri & Weinberg, 2009; Zeisberg & Neilson, 2009). These elongated spaces and cracks or clefts in the Rbm were not empty; they frequently contained cells, and these cells I found were positive for mesenchymal markers (S100A4 and vimentin) and also for a proteolytic enzyme MMP-9 which increases in abundance during the process of EMT. MMPs are thought to assist cell migration by dismantling underlying basement membrane as explained in Chapter 4.

Even though I have shown that these cells in the Rbm and basal epithelium are positive for mesenchymal markers, these data in themselves do not confirm that these cells are of epithelial origin. Therefore to confirm the epithelial origin of these cells in the Rbm, I needed to undertake double immunostaining with epithelial and mesenchymal markers.

The second major issue with the study presented in Chapter 4 was that I needed to address whether our data was confounded by infiltrating inflammatory cells that may carry epitopes for MMP-9 and S100A4. To refute this I needed to immunostain biopsies from COPD patients for neutrophils, macrophages, fibroblasts, CD4+ and

CD8+ lymphocytes, B cells and perhaps most importantly dendritic cells, as S100A4 is also expressed by dendritic cells (Boomershine et al., 2009).

Therefore, to confirm the epithelial origin of the S100A4 stained cells in the Rbm I double-stained biopsies from COPD patients for the epithelial marker cytokeratin and the mesenchymal marker vimentin. To address the potential for our S100A4 staining data to be confounded by infiltrating inflammatory cells we stained representative sections from the same tissue blocks for neutrophil elastase (neutrophil marker), CD68 (macrophage and mature fibroblast marker) (Beranek, 2005; Inoue et al., 2005), CD4+/CD8+, CD19 as a B-cell marker (Bertrand et al., 1997) and CD11c for potential dendritic cells (K. F. Chung & Adcock, 2008), and compared the number of cells staining for these markers *versus* the number staining for the S100A4 epitope.

5.2 Material and methods

Details are given in Chapter 3.

5.2.1 Biopsy analysis and measurements

This comparison involved bronchial biopsy sections from 10 COPD current smokers only. This analysis was an empirical semi-quantative analysis, as cellular changes in the Rbm were confirmed in the previous Chapters. For this analysis I had one best representative biopsy section from 10 different sections from COPD current smokers having maximum cellularity and assessed for different markers (as explained above) and results are presented as percentage of cells stained for different markers compared to S100A4, details are given below.

5.3 Results

Details of the subjects involved in this study are given in Chapter 4.

5.3.1 Confirming epithelial origin of cells stained for S100A4 in the Rbm

To confirm the epithelial origin of cells in the Rbm I double stained bronchial biopsy sections from COPD current smokers for cytokeratins (epithelial marker) and vimentin (another mesenchymal marker). This analysis demonstrated that 87 % of total cells double-stained for both cytokeratin and vimentin in the Rbm, while the rest of the cells were positive for vimentin but not for cytokeratin, presumably because these cells had lost epithelial markers during development of EMT (Figure 5.1).

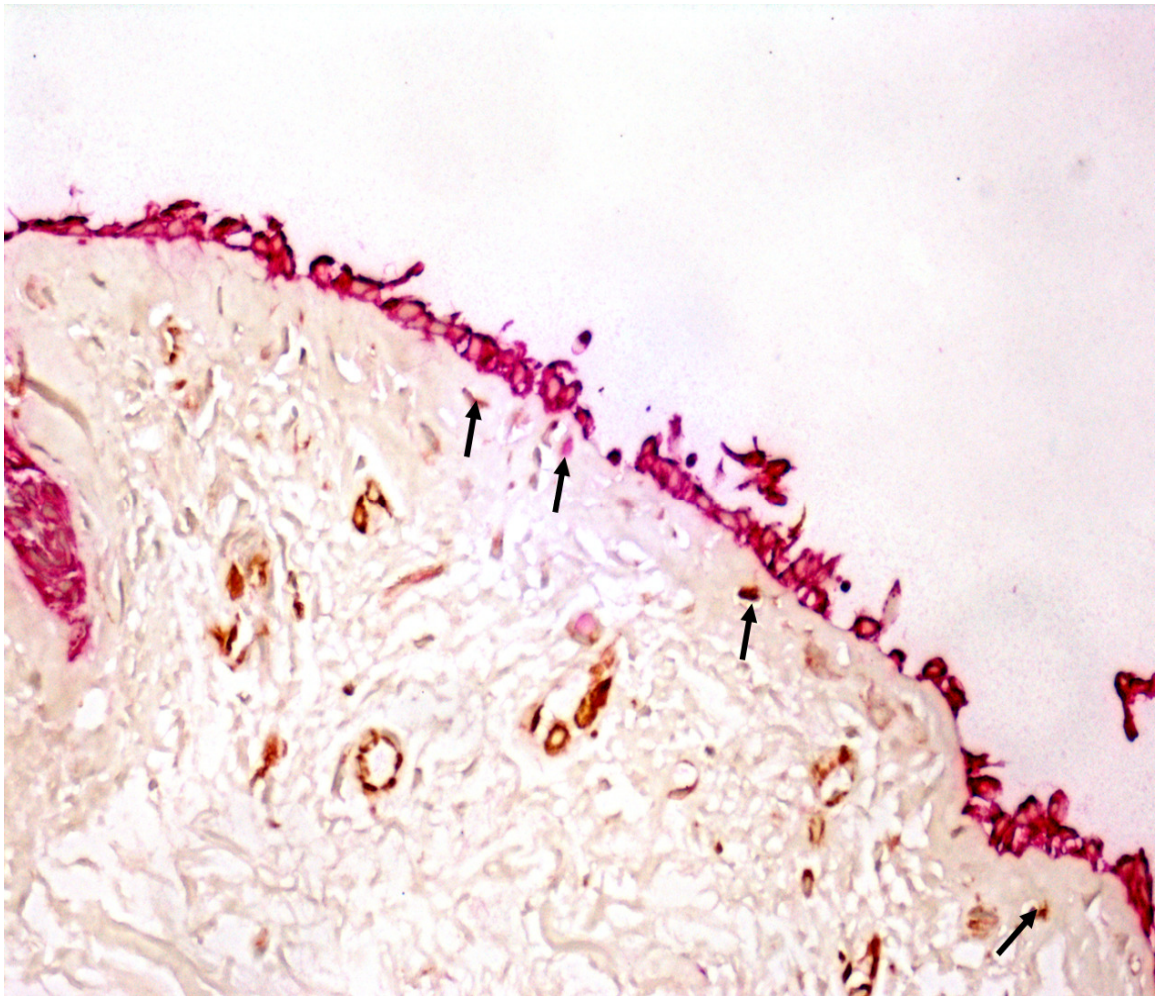


Figure 5.1: Bronchial biopsy specimen from a COPD current smoker, black arrows showing cells in the Rbm stained for both cytokeratin (pink or red; epithelial marker) and vimentin (brown; mesenchymal marker). Original magnification, x400

5.3.2 Comparative analysis of S100A4 staining with that for CD4+/CD8+ T lymphocytes and CD19 for B-cells in Rbm

To address the potential for our data to be confounded by infiltrating CD4+/CD8+ T lymphocytes and/or B-cells in the Rbm, I stained and assessed representative biopsy sections from COPD current smokers for CD4+ /CD8+ T lymphocytes and CD19 (B-cell marker) in the Rbm and basal epithelium.

This comparison demonstrated that in the epithelium no cells stained for CD4 or CD19, while approximately 4 % cells stained positive for CD8 whereas 68 % of total cells stained for S100A4 in the basal epithelium.

In Rbm no cells stained for CD4 or CD19 and approximately 3 % cells stained positive for CD8 whereas 97 % of total cells stained for S100A4. This analysis suggested that there are very few T or B lymphocytes within the Rbm or airway epithelium (Figure 5.2).

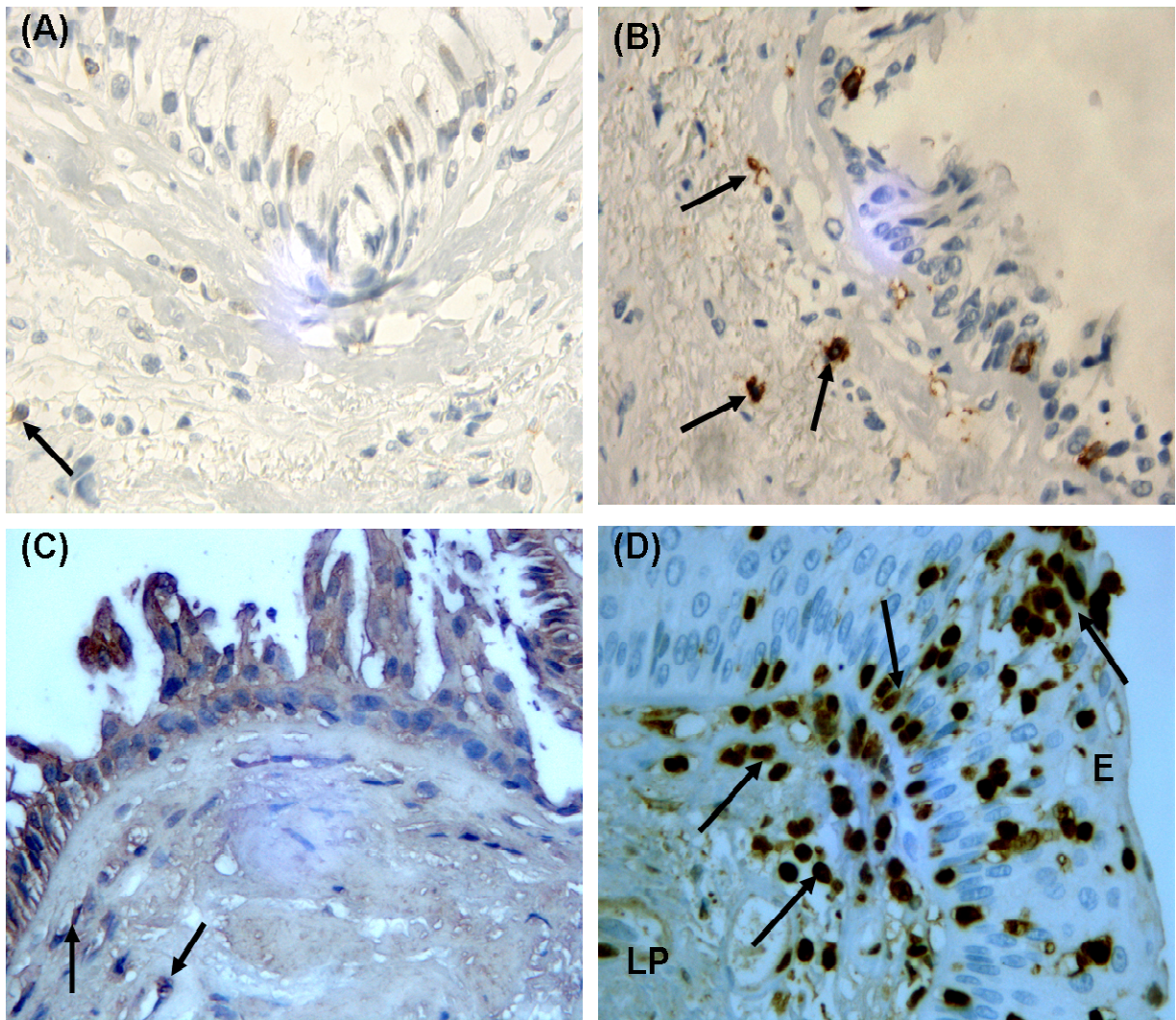


Figure 5.2: Bronchial biopsy specimen from a COPD current smoker, black arrows showing cells positive for: (A) CD4, mainly in the lamina propria (B) CD8, mainly in the lamina propria with very few cells +ve in the basal epithelium or Rbm (C) CD 19 (B-cell marker), mainly in the lamina propria (D) S100A4 stained cells, in the basal epithelium and Rbm, but also in the lamina propria, **E** (epithelium), **LP** (lamina propria). Original magnification, x630

5.3.3 Comparative analysis of S100A4 staining with that for dendritic cells

To address this question I stained and assessed matched representative biopsy section from COPD current smokers for CD11c (human dendritic cell marker) (Takano et al., 2005) and S100A4. This comparative analysis revealed that in the Rbm only 3.3 % of the total number of cells were stained for CD11c whereas 96.6 % were stained for S100A4, while in the basal epithelium only 1.5 % of the total number of cells stained for CD11c while 65 % of total cells stained for S100A4 (Figure 5.3). This analysis demonstrated that there were very few dendritic cells in the Rbm and epithelium compared to S100A4 positive cells.

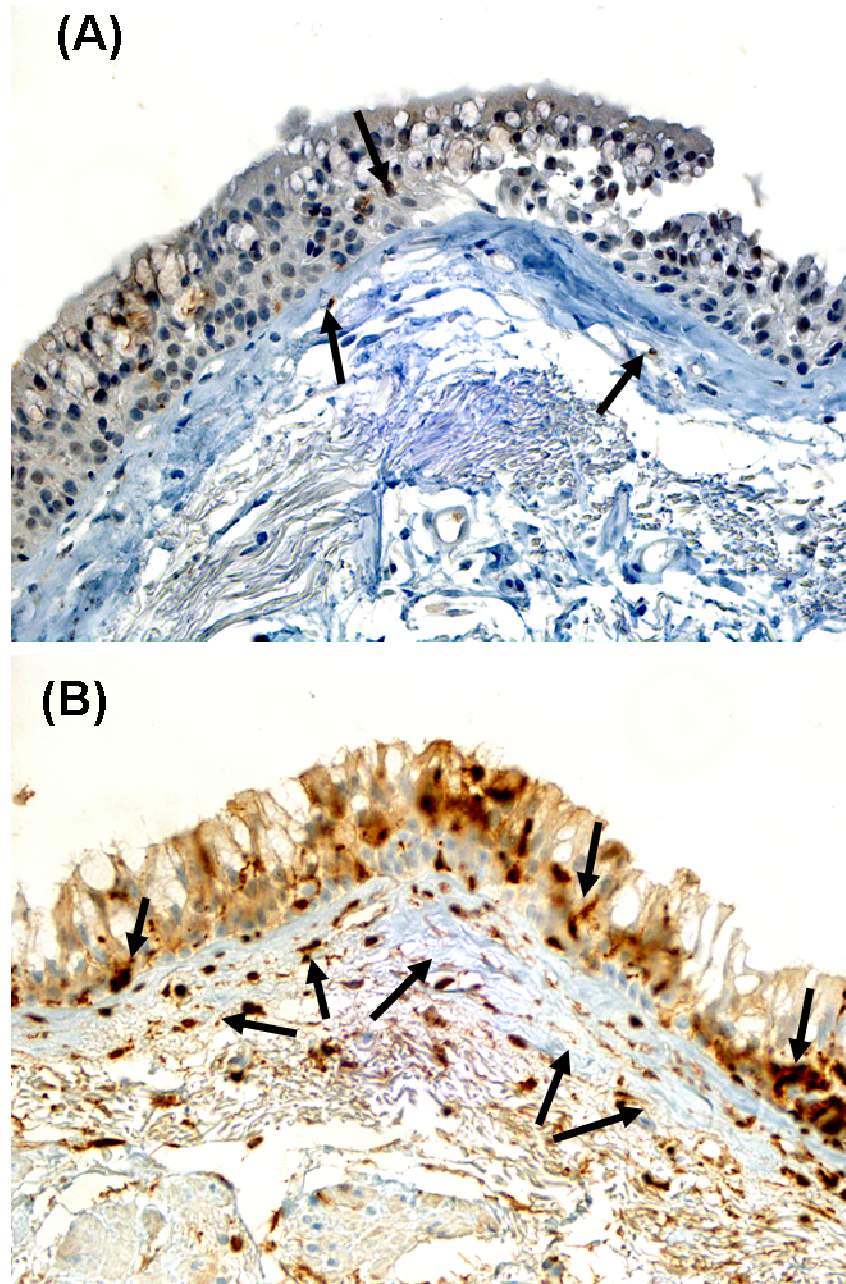


Figure 5.3: Bronchial biopsy specimen from a COPD current smoker, black arrows showing cells in the Rbm stained for: (A) CD11c (dendritic cell marker) and (B) S100A4 (mesenchymal marker). Original magnification, x400

5.3.4 Comparative analysis of MMP-9 and S100A4 staining with that for polymorphonuclear neutrophils (PMNs) and macrophages

To address the potential for our data to be confounded by infiltrating inflammatory cells that may carry epitopes for MMP-9 and S100A4, I stained and assessed, representative sections from the same tissue blocks (as above) for neutrophil elastase (neutrophil marker) and CD68 (macrophage and mature fibroblast marker) (Beranek, 2005; Inoue et al., 2005; Kunz-Schughart et al., 2003). This comparison demonstrated that no cells stained for neutrophil elastase or CD68 whereas again 67.6 % (same data as above) of total cells stained for S100A4 in the basal epithelium. In the Rbm no cells stained for neutrophil elastase or CD68 whereas 96.9 % (as given above) of total cells stained for S100A4 (Figure 5.4).

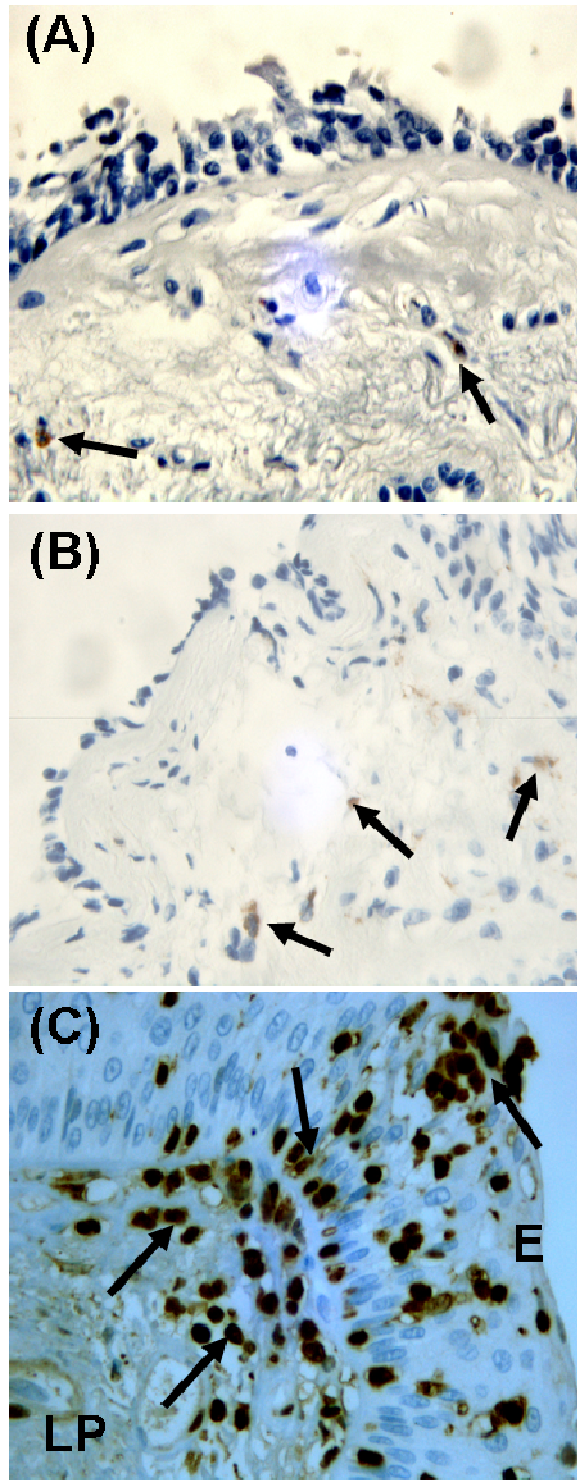


Figure 5.4: Bronchial biopsy specimen from a COPD current smoker, black arrows showing cells positive for: (A) neutrophil elastase (neutrophil marker) mainly in the lamina propria; and (B) CD68 (macrophage and mature fibroblast marker) also mainly in the lamina propria; (C) S100A4, where there are frequent S100A4 +ve cells in the basal epithelium and Rbm, **E** (epithelium), **LP** (lamina propria). Original magnification, x630

5.4 Discussion

In the previous Chapter, I demonstrated that EMT could well be an active process in COPD airways. I showed that the Rbm in COPD is highly fragmented with cells staining for S100A4 and MMP-9. In this Chapter, I have followed this up by demonstrating that these cells in the Rbm are of epithelial origin, they are not infiltrating macrophages or fibroblasts, nor CD4+/CD8+ T lymphocytes or B-cells or dendritic cells. Thus, my data has not been confounded by infiltration by inflammatory or immune cells. This has been a criticism of my work when it was presented at international meetings and from journal reviewers.

To address the epithelial origin of cells in the Rbm I double stained biopsy sections from COPD current smokers for the epithelial marker cytokeratin, as well as the mesenchymal marker vimentin. This analysis confirmed that the cells in the Rbm are of epithelial origin as well as demonstrating a co-staining mesenchymal epitope. Had these cells been negative for cytokeratin, this would have been difficult to interpret as during development of EMT epithelial cells loose epithelial markers as they gain mesenchymal markers. Therefore, a cell which has gone through a complete transition will not express epithelial markers. I find the presence of cells staining with cytokeratin and still in the epithelium staining for vimentin especially suggestive of EMT, but it is not surprising that there are more cells staining for vimentin than cytokeratin. Indeed, this is likely to be another signal that EMT is active in this epithelium ie that epithelial cells are loosing epithelial markers.

I found that there were very few CD8+ T lymphocytes and no CD4+ T cells or CD19 B-cell cells in the Rbm and basal epithelium. There were some cells positive for CD4 and CD19, but these were mainly in the lamina propria region and not in the basal epithelium or Rbm as such. I also confirmed that my data was not confounded by infiltrating dendritic cells for which I stained sections for CD11c and found that there were very few such cells in the basal epithelium and Rbm compared to S100A4. I also confirmed that they are not infiltrating neutrophils, macrophages or fibroblasts, and most of the cells positive for these markers were observed in the lamina propria below the Rbm, and not in the Rbm itself, nor in the basal epithelium in contrast to S100A4 positive cells which were very frequent in the Rbm.

In summary I have demonstrated that the cells in the Rbm are of epithelial origin, and they are not infiltrating macrophages, fibroblasts, CD4+/CD8+ T lymphocytes, B-cells nor dendritic cells. These data confirm that EMT is indeed likely to be an active process in the airways in smokers and COPD patients, and that my data presented in Chapter 4 was not confounded by infiltrating inflammatory or immune cells.

Chapter 6

Effect of Inhaled Corticosteroids on Rbm Fragmentation: potential for the amelioration of EMT

6.1 Introduction

Inhaled corticosteroids (ICS) are widely used for treatment of COPD even though their efficacy is still a matter of debate (Telenga et al.). However, several studies have shown that with short term use of ICS lung function is improved in COPD, long term use reduces the frequency of exacerbation in those with severe disease. Even so it is still likely that some aspects of airway and lung parenchymal inflammation and structural changes involved in COPD are less responsive to ICS than in asthma (Chanez et al., 2004; Telenga et al.). Bronchial biopsies have been suggested as a useful tool to investigate the anti-inflammatory effects of ICS in both asthma and COPD, but there are very few studies reporting the effects of ICS on airway inflammation and even fewer on structural changes or airway remodelling in COPD using bronchial biopsies. The reality is that we really don't know much about these processes and drug efficacy on them, especially in COPD (Chanez et al., 2004).

Hattotuwa and colleagues reported a significant reduction in CD4/CD8 cell ratios in the epithelium, and in sub-epithelial mast cells in bronchial biopsies obtained from COPD patients in the active ICS arm treated with fluticasone propionate (Hattotuwa et al., 2002). Reid and et al reported from observations made in bronchial biopsies and BAL from COPD patients, that fluticasone propionate reduced BAL neutrophils and epithelial cell numbers and CD68+ macrophages, CD8+ lymphocytes and mast cells in bronchial biopsies, but interestingly increased neutrophils in bronchial biopsies (D. W. Reid & et al., 2008). In another study it was reported that three month treatment with fluticasone propionate significantly decreased mucosal mast

cells and increased neutrophils in biopsies from COPD patients (Gizycki et al., 2002).

The data on effects of ICS on structural changes in COPD is very sparse, and there are no studies examining effects of ICS on epithelium. Even the baseline Rbm thickness in COPD is in doubt, with some studies reporting abnormally thick Rbm and some not (Chanez et al., 2004; Liesker et al., 2009; Postma & Timens, 2006). The effects of ICS on the Rbm have not been specifically studied in COPD. Zanini and et al, however reported that bronchial vascular remodelling may have potential for change with ICS but need further longitudinal studies to confirm that (Zanini & et al., 2009).

As described in previous chapters, it is likely that in COPD the epithelium may get damaged by cigarette smoke and remodelling may occur in response to cigarette smoke-induced damage to the airways and related inflammation (Pera & et al 2010; Hu & et al 2009), but the precise mechanisms and details structural changes are poorly described in the literature. As already discussed at length, new emerging explanation for remodelling in chronic lung diseases is epithelial mesenchymal transition (EMT) which involves transition of airway epithelial cells to a mesenchymal phenotype and movement through the Rbm to the lamina propria, causing Rbm fragmentation (for details please see Chapter 1, 2, 3 and 4) (Peter K. Jeffery, 2004; Brigham C. Willis et al., 2006).

In previous Chapters, I have shown that the Rbm in COPD is highly fragmented, with elongated spaces or cracks, which I have termed “clefts”. These, I believe, are the hallmark of EMT, strongly suggested by previous non-airway models of EMT described in detail in the literature (Acloque et al., 2009; R. Kalluri, 2009; Raghu Kalluri & Neilson, 2003; R. Kalluri & Weinberg, 2009; Zeisberg & Neilson, 2009). In the previous chapters I described a comprehensive cross-sectional study looking at the potential for EMT in COPD and I concluded that EMT is likely to be an active process in COPD but a key question which still remains unanswered is whether ICS have any potential for amelioration or even reversal of EMT in COPD. Therefore, I hypothesized that Rbm fragmentation, a hallmark of EMT is normalised by aggressive ICS therapy in patients with COPD. To address this question I used

material from a longitudinal study (D. W. Reid et al., 2008) in which COPD subjects (current smokers and ex-smokers) entered a double blind randomised controlled trial comparing fluticasone propionate (0.5 mg inhaled twice daily) with identical placebo for 6 months.

6.2 Hypothesis

I hypothesized that Rbm fragmentation, a hallmark of EMT is normalised by aggressive ICS therapy in patients with COPD.

6.2.1 Aim

To assess the effect of fluticasone propionate on Rbm fragmentation, which I have regarded as the hallmark of EMT.

6.3 Methods and materials

The group demographics of subjects who participated in the study are presented in the table given below. As the number of samples available for analysis for different markers varied slightly due to loss during processing, the number of samples included in each comparison is detailed.

Table 5: Demographic and lung function data for subjects.

Groups (numbers)	FP (22)	Placebo (10)
GOLD I/GOLD II♣	10/12	4/6
Male/female	8/14	3/7
Age (years)	60(46-69)	62(52-69)
COPD-CS / COPD-ES	12/10	3/7
Smoking (pack years)	42(18-150)	54(22-147)
FEV1% predicted (Post BD)★	77(55-112)	77(54-94)
FEV1/FVC % (Post BD)★	58(41-66)	56(38-67)

Data expressed as median and range

★ Post BD values after 400µg of salbutamol

♣ Diagnosis of COPD was made according to GOLD guidelines

6.3.1 Biopsy analysis

All slides were coded and randomised by an independent person (lab manager Steven Weston), and then counted (as described in Chapter 3 and 4) by me in a single batch while fully blinded to subject diagnosis and phase of the study with quality assurance on randomly selected slides provided by a professional academic pathologist (Professor HK Muller).

6.3.2 Statistical analysis

Wilcoxon two related-samples test was used to test the effect of ICS and placebo. Associations between variables were assessed using Spearman's rank test. Statistical analyses were performed using SPSS 15.0 for Windows, 2003, with a two-tailed P -value ≤ 0.05 being considered statistically significant. I also performed power and sample size calculations for this analysis. This test showed that number of subjects smaller than 15 were adequate to find differences for Rbm fragmentation before and after treatment with alpha error 5% and beta error 80%. Richmond et al showed in studies done with airway biopsies (cellular quantitative studies) that sample size of 15 are adequate (Richmond, Booth, Ward, & Walters, 1996).

6.4 Results

6.4.1 Rbm fragmentation

The length of the individual “clefts” was measured, summed and results expressed as percentage of the total Rbm length to give a comparable measure of the degree of Rbm fragmentation for each individual. The degree of fragmentation decreased significantly ($p=0.03$) in the treatment arm (fluticasone propionate) (Figure 6.1) and when compared to placebo ($p=0.4$). At the end of treatment, there was no longer any difference between normal controls and COPD subjects who had received ICS ($p=0.7$) for Rbm fragmentation, while in the placebo group fragmentation was still increased above normal compared ($p=0.02$). Thus, inhaled steroids normalised Rbm fragmentation over 6 months of treatment (Figure 6.2).

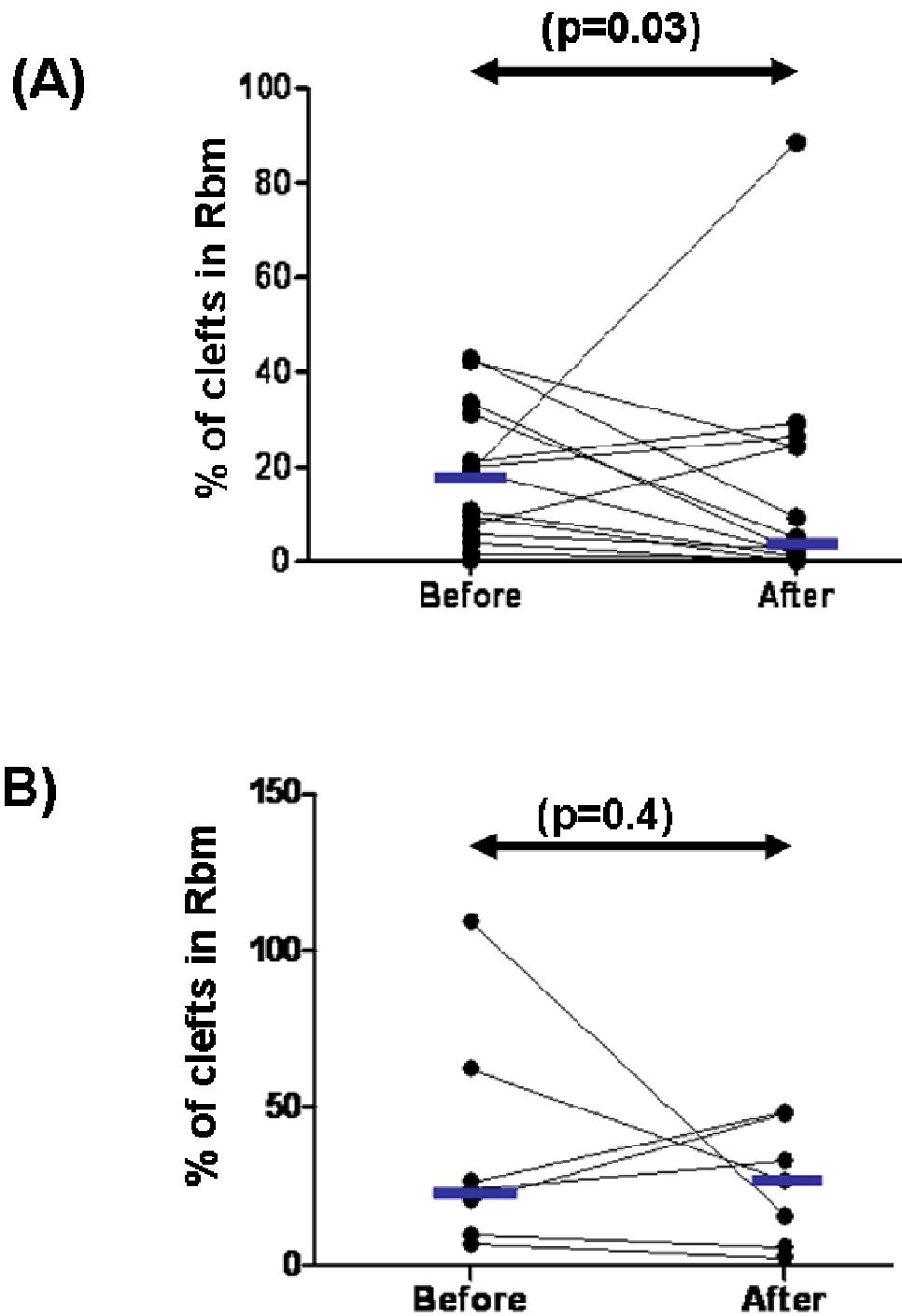


Figure 6.1: The percentage decrease in clefts (or Rbm fragmentation) in the Rbm per mm of Rbm on treatment with **(A)** fluticasone propionate ($p=0.03$) and **(B)** placebo ($p=0.4$), the horizontal bars represent the medium.

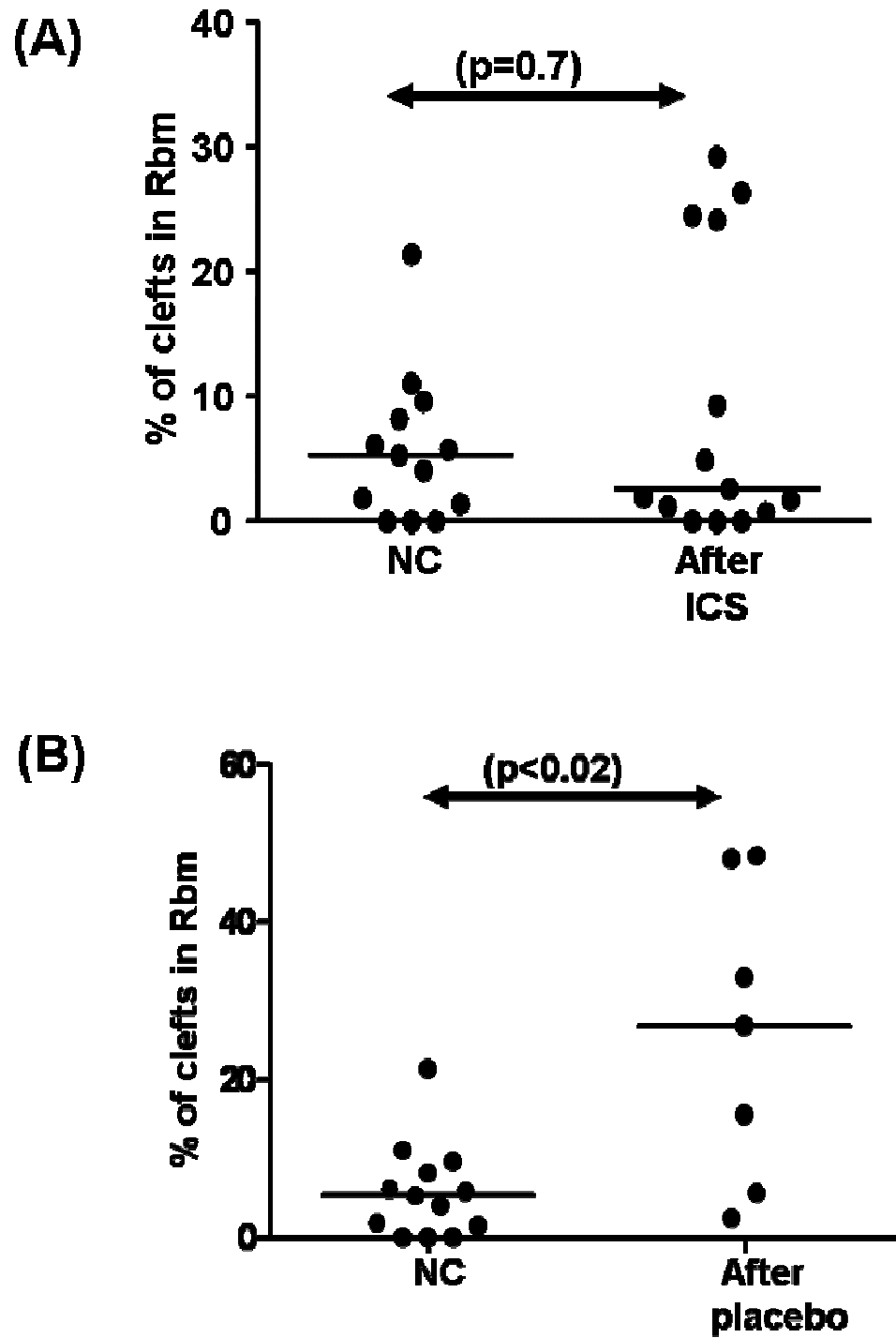


Figure 6.2: The Rbm fragmentation (given by % of clefts) is normalised on treatment with (A) ICS (fluticasone propionate) ($p=0.7$), but not with (B) placebo ($p=0.02$), compared to normal controls, the horizontal bars represent the medium.

6.5 Discussion

This is the first study reporting the potential of fluticasone propionate for affecting Rbm fragmentation and hence EMT. But since I am the first to describe fragmentation and potential EMT in COPD airway biopsies this is perhaps not surprising! In this study I have shown that Rbm fragmentation, which my reading of the literature has brought me to believe is a hallmark of EMT (Acloque et al., 2009; R. Kalluri, 2009; Raghu Kalluri & Neilson, 2003; R. Kalluri & Weinberg, 2009; Zeisberg & Neilson, 2009) is responsive to ICS, and indeed effectively normalised by it, at least on average although there was some variation in response.

ICS are considered to be the most effective therapy for the treatment of chronic inflammatory disorders such as asthma, although they may be rather less effective in control of inflammation in other inflammatory airway disorders such as COPD and cystic fibrosis. However, there are studies showing that ICS have been beneficial in controlling inflammation to some extent in COPD, but there are hardly any studies reporting effects of ICS on structural changes or airway remodelling in COPD using bronchial biopsies apart from one study reporting potential for bronchial vascular remodelling (Gizycki et al., 2002; Hattotuwa et al., 2002; D. W. Reid et al., 2008; Zanini et al., 2009). In asthma Ward et al from our group have shown that Rbm thickness does decrease significantly on 12 months treatment with fluticasone propionate, closely related to the slow phase of improvement in BHR (bronchial hyper-responsiveness) that occurs, showing potential for ICS in airway remodelling more generally (Ward et al., 2002).

EMT is well described in post lung transplant in chronic airway rejection (known as BOS, bronchiolitis obliterans syndrome) and it is suggested that EMT may be a potential link between inflammatory injury and airway remodelling in this situation (Borthwick et al., 2009; Ward et al., 2005). However, there have been no previous studies reporting EMT in COPD to the best of my knowledge. Even so I believe that I have now provided evidence that EMT is an active process in COPD airways (as explained in Chapter 1, 4 and 5), and have now taken this one step further in showing that this is positively affected by ICS. This is a key issue, for although ICS

do seem to have positive clinical effects in COPD, the science behind this has been somewhat opaque.

On a negative note, I accept that this study is limited by the fact that I haven't undertaken comprehensive longitudinal analysis with direct cellular markers of EMT as described in Chapter 4, and this was due to time limits I had with my PhD submission. Even so, I have used Rbm fragmentation, generally regarded as a central hallmark manifestation of active EMT as the outcome measure in this analysis, and will follow it up with future analysis of Rbm cellularity and specific cellular EMT markers.

In summary I have shown that fluticasone propionate has potential for normalising EMT, using quantitation of Rbm fragmentation as the illustrative manifestation of the process in COPD airways. I need now a comprehensive longitudinal follow-up study with a wider range of markers of EMT (for e.g. S100A4, vimentin, cytokeratins) to confirm and extend these preliminary findings.

Chapter 7

HDAC2 in COPD

7.1 Introduction

As already described in detail in Chapter 2, COPD is characterized by progressive inflammation of the airways (and destruction of the lung parenchyma) mediated by increased expression of inflammatory genes, in response to noxious particles, gases and especially tobacco smoke (Barnes, 2003). In COPD a specific pattern of airway inflammation is mainly characterized by increased numbers of neutrophils, macrophages and T-lymphocytes, predominantly cytotoxic (CD8+) cells (Barnes et al., 2005; Saetta et al., 1993; Saetta et al., 1998; Saetta et al., 1997; Saetta, Turato, Maestrelli, Mapp, & Fabbri, 2001). The increased expression of these inflammatory genes is regulated by acetylation of core histones around which DNA is wound, allowing access of pro-inflammatory transcription factors to transcription-regulatory sites. On the other hand these activated genes are switched off by deacetylation of these histones (Chapter 2) (Barnes et al., 2005). Examples of the transcription factors involved in this regulatory system in airway diseases are nuclear factor-Kappa B (NF- κ B), activator protein-1 (AP-1) and also the activated glucocorticoid receptors (Barnes, 2006d). These are all at least partly controlled by this histone acetylation/deacetylation mechanism.

COPD responds relatively poorly to therapeutic corticosteroids, even though steroids have been shown to have effects both in the short term and long term use. As discussed in Chapter 2 there is emerging evidence that corticosteroids resistance in COPD is due to decrease in histone deacetylase -2 (or HDAC-2) (Barnes, 2006c; Ito et al., 2001). Though exact mechanisms are still not clearly understood, it is suggested that it may involve modulation of HDACs by nitrosylation on distinct tyrosine residues in response to tobacco smoke (Barnes et al., 2005).

Since 1960, it has been known that acetylation of DNA-associated histone proteins and remodelling of the tightly packed chromatin structure is associated with

induction of genes (Littau et al., 1965). Histone and chromatin remodelling is central to gene expression and regulation through the process of acetylation, deacetylation and also methylation, though this is even less understood (Barnes et al., 2005; Rice & Allis, 2001). Research regarding the exact role of histone acetylation and deacetylation in chronic inflammatory disease is only in its infancy, and even more so in COPD, where the picture has probably been made rather over-simplistic, as indeed the degree of absolute ICS insensitivity has been exaggerated. However, there is significant body of literature (as explained in Chapter 2) suggesting that expression and activity of anti-inflammatory HDAC2 are reduced in COPD lungs, airways and alveolar macrophages and becomes worse with severity of the disease (Barnes, 2006c; Ito et al., 2001).

The methodology that has been used was not comprehensive enough to identify the changes in total cellularity in the lamina propria and largely dependent on molecular RNA quantitation and protein analysis which could not take into account relative differences in cellular profiles in airway tissues in different disease and control groups, where biases could arise due to differences in total and differential cellularity. There has been a serious lack of comprehensive biopsy studies to confirm the extent of suppressed HDAC2 levels by immunostaining within the airways of COPD; which can take into account cell numbers and type, its reversibility with ICS or smoking cessation. Therefore, I designed a detailed cross-sectional study and used material collected in a longitudinal study (D. W. Reid et al., 2008), for looking in detail at the status of HDAC2 expression in COPD airways.

7.2 Hypothesis

I hypothesized that the current literature is correct in that HDAC2 is down-regulated in COPD airways, and that these reduced HDAC2 levels are normalised by aggressive ICS therapy and also smoking cessation in patients with COPD.

7.2.1 Aims

1. To confirm the extent of suppressed HDAC2 by immunostaining airway biopsies from COPD current smokers and also from normal lung function smokers to understand whether changes in HDAC2 are solely related to COPD or smoking or both.
2. To confirm the potential of smoking cessation to raise HDAC2 expression by comparing airway biopsies from COPD ex-smokers *versus* current smokers with COPD, and the potential for HDAC2 expression to be normalised by aggressive ICS therapy.

7.3 Methods and materials

7.3.1 Subjects and study design

The studies involved both detailed cross-sectional and longitudinal analyses. In the cross-sectional study, 17 current smokers with established COPD (CS), 16 current smokers with normal lung function (NS), 17 ex-smokers with COPD (ES) and 15 normal healthy, never-smoking controls (NC) were recruited for bronchoscopy and airway biopsy. In longitudinal analysis the COPD subjects further entered a double blind randomised controlled trial comparing fluticasone propionate (0.5 mg inhaled twice daily) with placebo for 6 months (details given in Chapter 3).

7.3.2 Bronchoscopy

Details are given in Chapter 3.

7.3.3 Processing of biopsies and immunostaining

Sections were stained with monoclonal antibodies: anti-HDAC2, together with a horseradish peroxidase (HRP) conjugated DAKO Envision + reagent for secondary antibody binding and colour resolution using *diaminobenzidine* (DAB) (details given in Chapter 3).

7.3.4 Biopsy analysis

Details are given in Chapter 3.

7.3.5 Statistical analysis

As data were not normally distributed (tested by kolmogorov smirnov & Shapiro Wilk test), a non-parametric ANOVA (Kruskal Wallis Test) was undertaken, and specific group differences then explored using the Mann Whitney U test. Wilcoxon two related-samples test was used to test the effect of ICS and placebo in the longitudinal study. Associations between variables were assessed using Spearman's rank test. Statistical analyses were performed using SPSS 15.0 for Windows, 2003, with a two-tailed P -value ≤ 0.05 being considered statistically significant (details are given in Chapter 3).

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7.4 Results (cross-sectional analysis)

The group demographics of subjects who participated in the study are the same as presented Chapter 4.

7.4.1 HDAC2 expression in the airway epithelium

HDAC2 expression in the airway epithelium (as measured by percentage area of epithelium stained for HDAC2) was not different between the groups ($p=0.7$, Figure 7.1 and 7.2).

7.4.2 Total cellularity of the lamina propria

There, was a significant reduction in total number of cells in lamina propria in COPD-CS compared to NC ($p=0.04$) (Figure 7.3 and 7.1). This total cell change strongly negatively correlated to smoking history ($R = -0.8$, $p<0.003$) (Figure 7.6). No association was found with lung function measurements.

7.4.3 HDAC2 positive cells in the lamina propria

Compared to NC there was a significant reduction in HDAC2 positive cells in the lamina propria in COPD-CS ($p=0.02$). Interestingly, normal lung function smokers had significantly more HDAC2 positive cells in the lamina propria compared to COPD-CS ($p<0.001$), ie smoking actually seems to be stimulating anti-inflammatory HDAC2. There were significantly more HDAC2 positive cells in COPD-ES compared to COPD-CS ($p=0.04$) (Figure 7.4 and 7.1); ie COPD-CS smokers were essentially normalised. No association was found with lung function measurements.

7.4.4 Percentage cell HDAC2 staining in the lamina propria

For percentage cell HDAC2 staining (ie taking into account total cell numbers) normal lung function smokers were significantly different from all other groups ($p<0.001$, with smoking again apparently stimulating anti-inflammatory HDAC2), but COPD-CS, ES and NC were quite similar ($p=0.42$) (Figure 7.5). No association was found with lung function measurements.

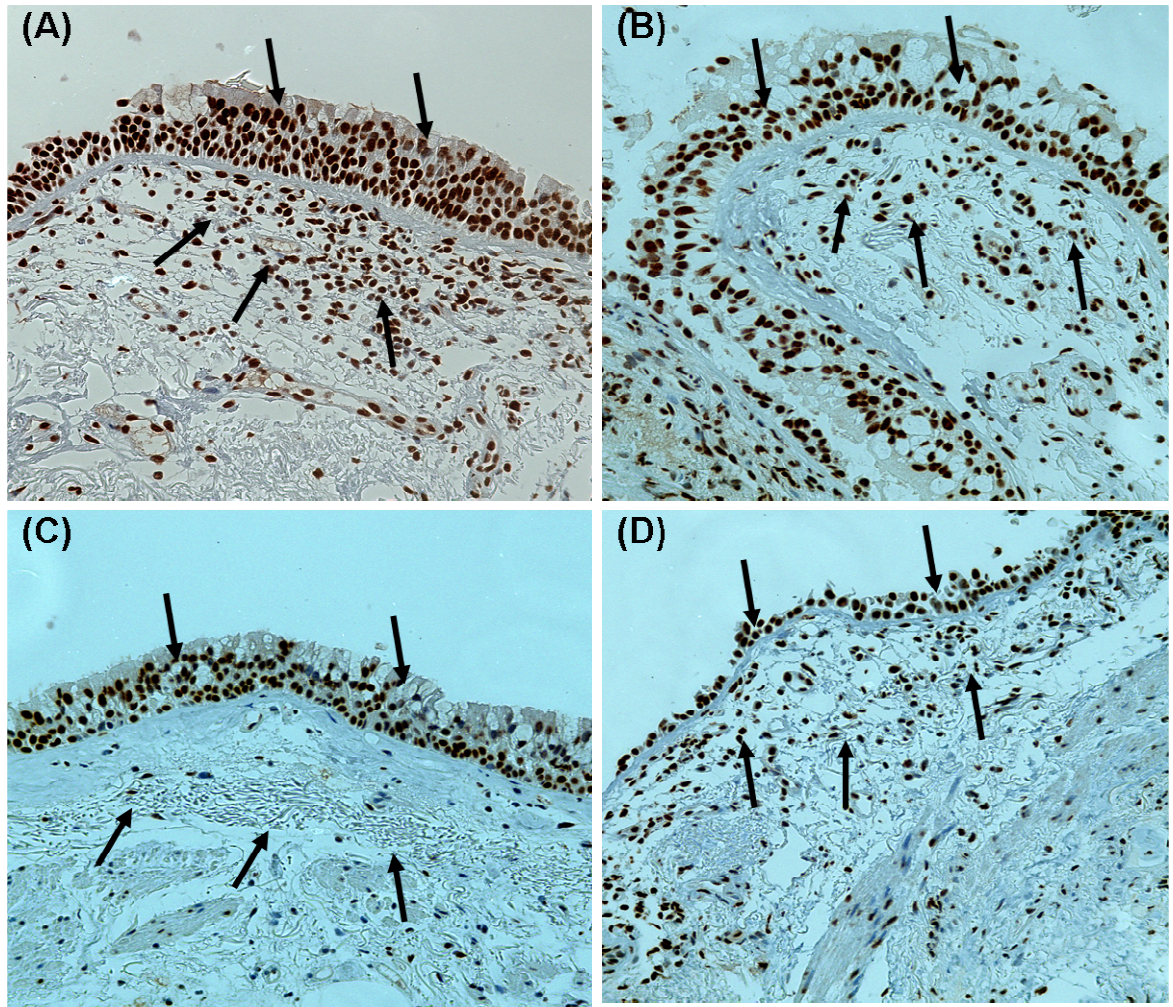


Figure 7.1: Bronchial biopsy section from (A) normal control; (B) normal lung function smoker; (C) COPD current smoker; and (D) COPD ex-smokers: (black arrows) brown staining indicates HDAC2 positive cells in the epithelium and in the lamina propria, *black arrows, also indicating decreased cellularity in the LP in (B) and (C).* Original magnification, x400.

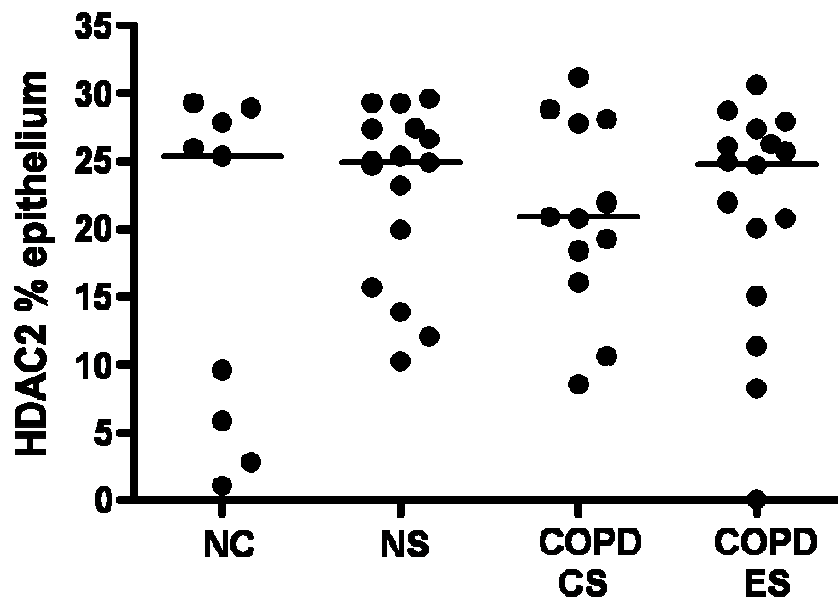


Figure 7.2: Percentage of **area** of epithelium stained for HDAC2 in COPD current smokers and ex-smokers, normal lung function smokers compared to normal controls with no significant difference between groups. Horizontal bars represent the median for each group.

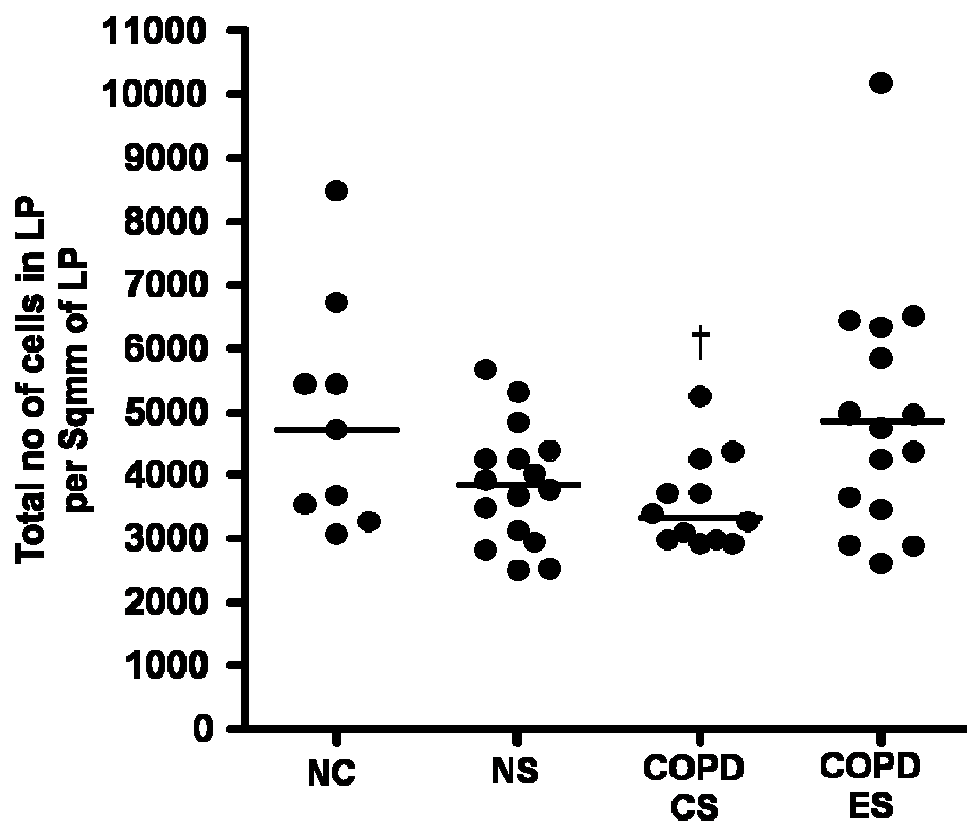


Figure 7.3: Total number of cells in the lamina propria per Sqmm of lamina propria;

†significant difference from NC (p=0.04).

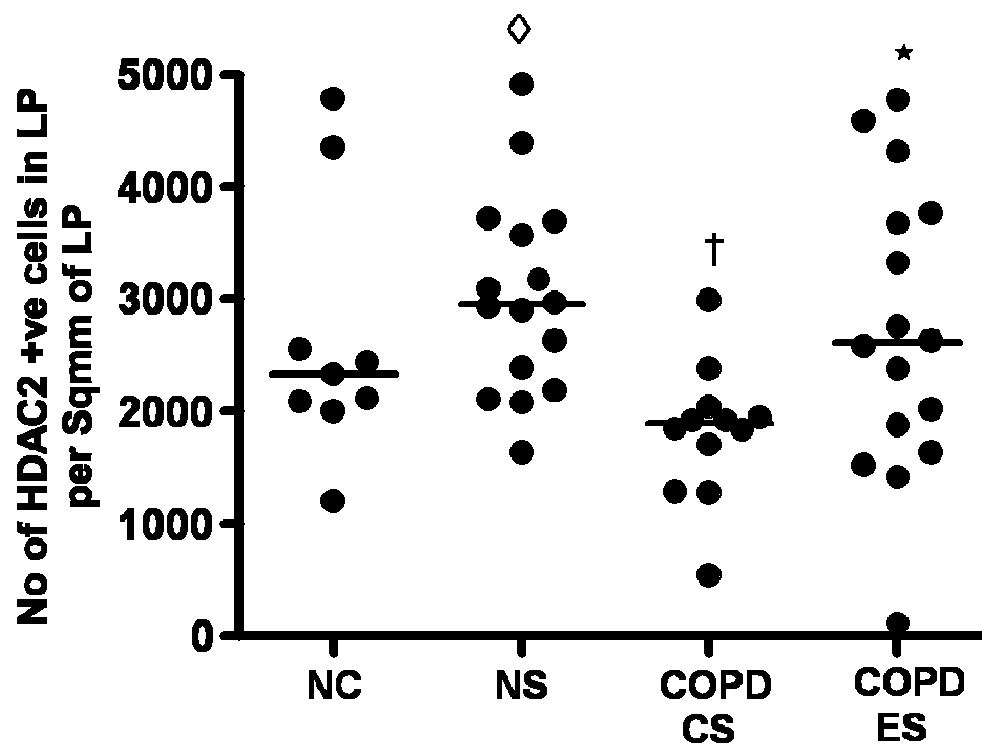


Figure 7.4: Number of HDAC2 positive cells in the lamina propria per Sqmm of lamina propria; †significant difference from NC ($p=0.02$); †significant difference from COPD-CS ($p<0.001$); ★significant difference from COPD-CS ($p=0.04$).

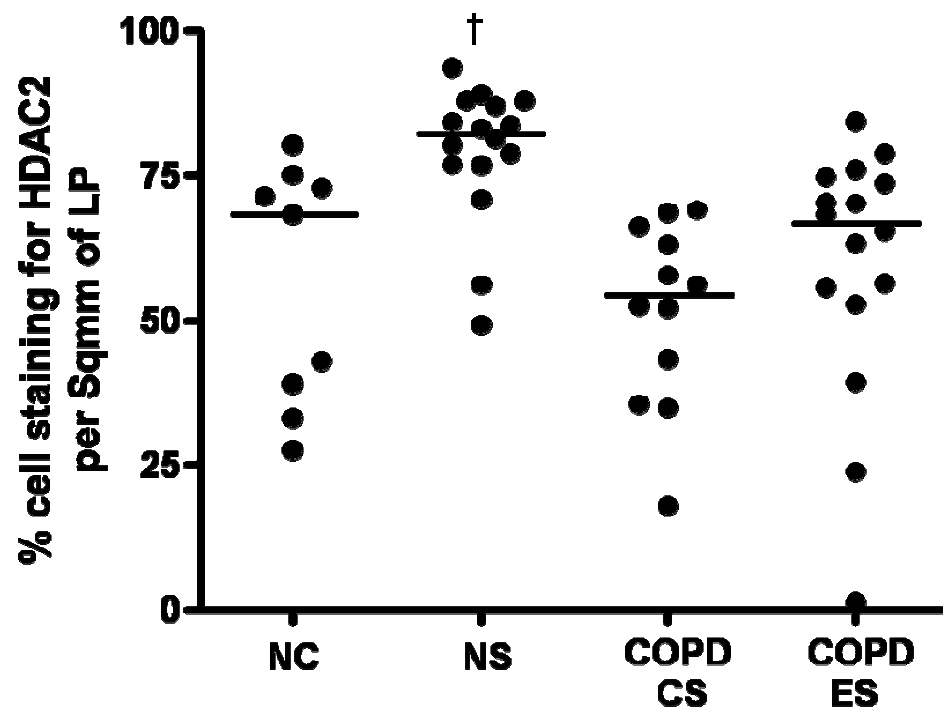


Figure 7.5: Percentage cell staining for HDAC2 in the lamina propria per Sqmm of lamina propria; †significant difference from COPD-CS, ES and NC ($p<0.001$).

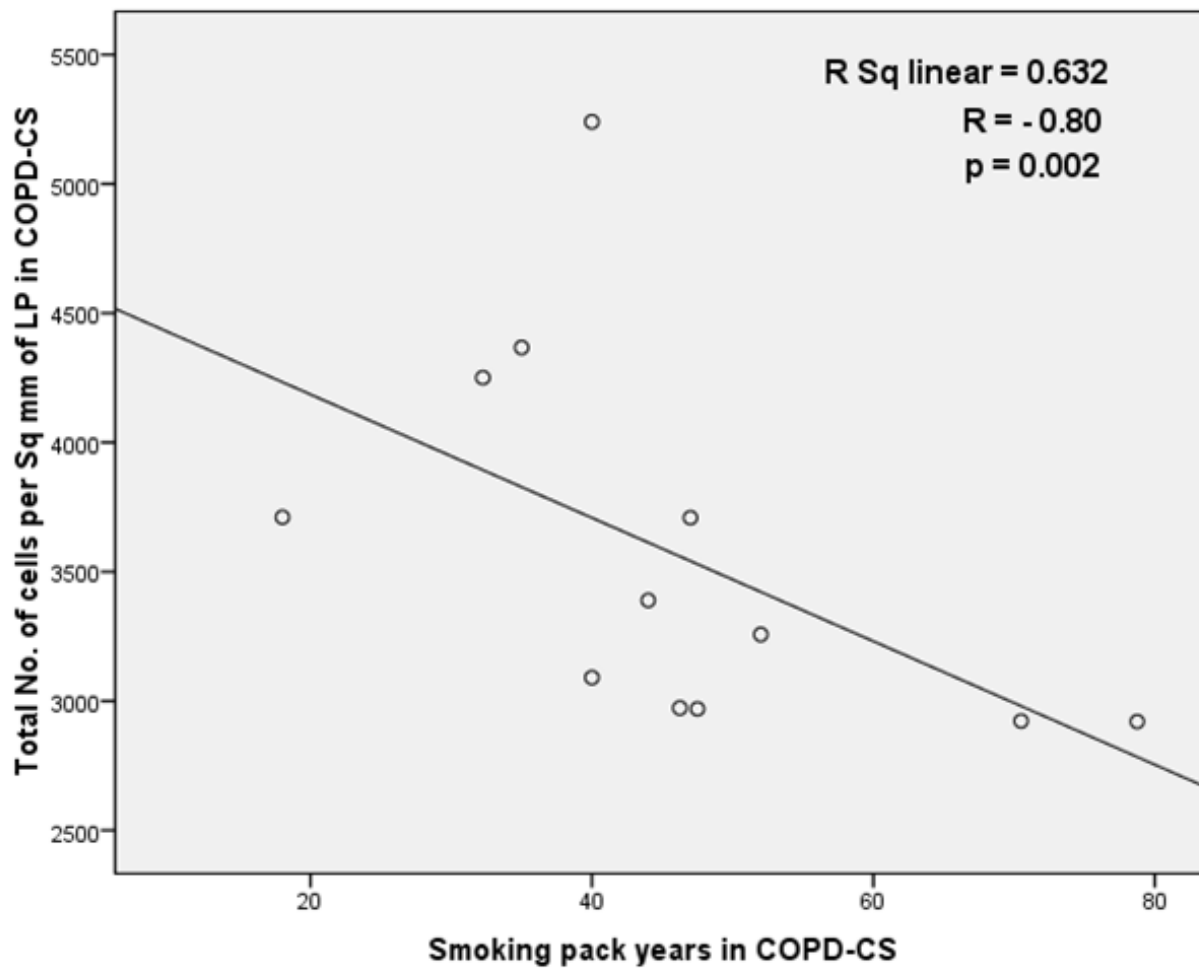


Figure 7.6: Correlation between total number of cells per Sqmm of lamina propria in COPD-CS and smoking (pack years).

7.5 Longitudinal ICS intervention study

The group demographics of subjects who participated in the study are the same as presented Chapter 6.

7.5.1 HDAC2 expression in the airway epithelium

Six months of treatment with fluticasone propionate (active arm; $p=0.2$, placebo; $p=0.5$) made no difference on HDAC2 expression in the airway epithelium (Figure 7.7).

7.5.2 HDAC2 positive cells in the lamina propria

There was no difference observed before *versus* after treatment for HDAC2 positive cells in the lamina propria (active arm, $p=0.5$ and placebo; $p=0.3$, Figure 7.8). I also observed that there was no change in the total number of cells in the lamina propria before and after treatment (active arm, $p=0.5$ and placebo; $p=0.8$, Figure 7.9). Thus, percentage cell HDAC staining was also not different before and after treatment (active arm, $p=0.5$ and placebo; $p=0.8$, Figure 7.10).

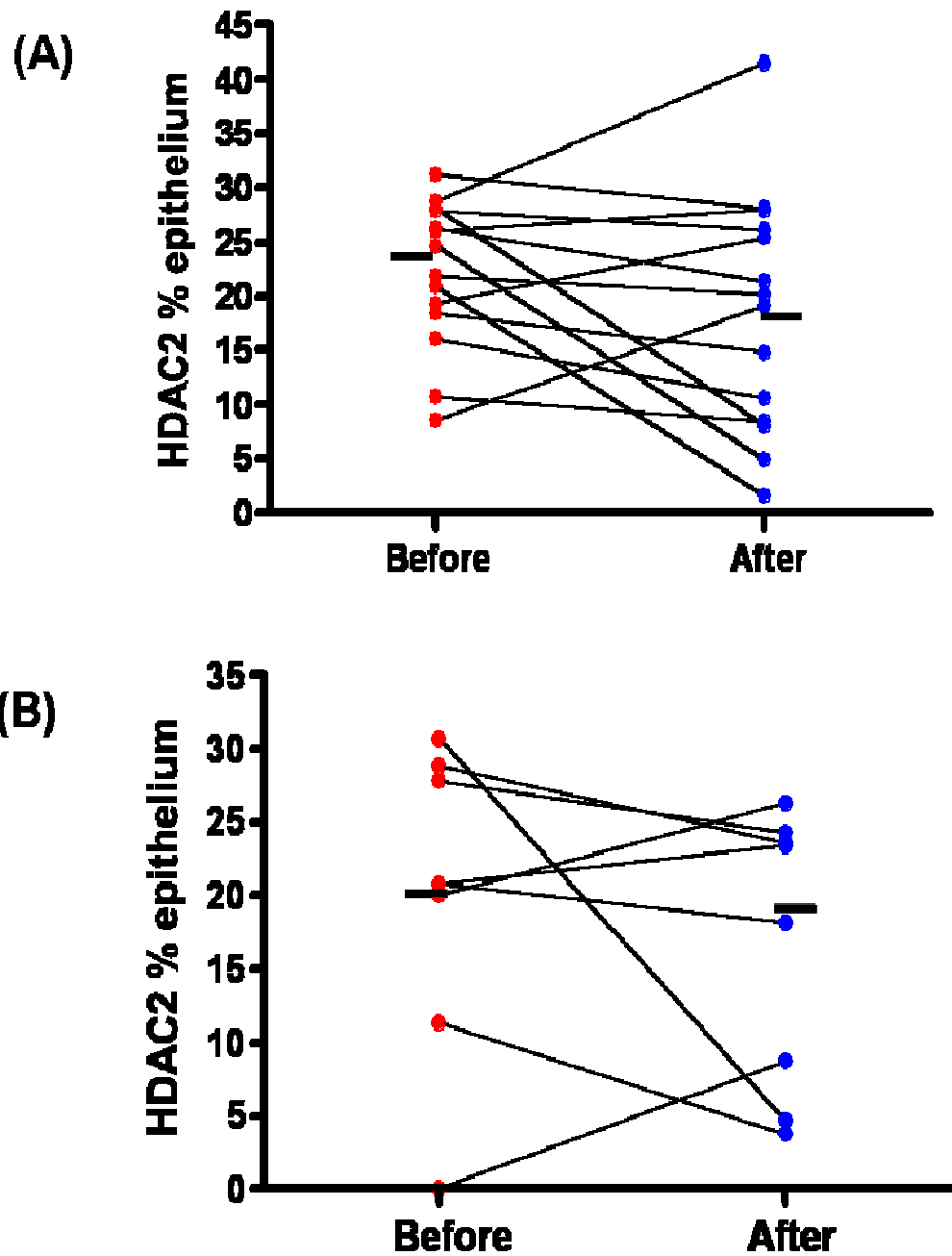


Figure 7.7: The percentage change in HDAC2 in the epithelium on treatment with (A) fluticasone propionate and (B) placebo, the horizontal bars represent the median. There was no significant difference comparing before and after treatments.

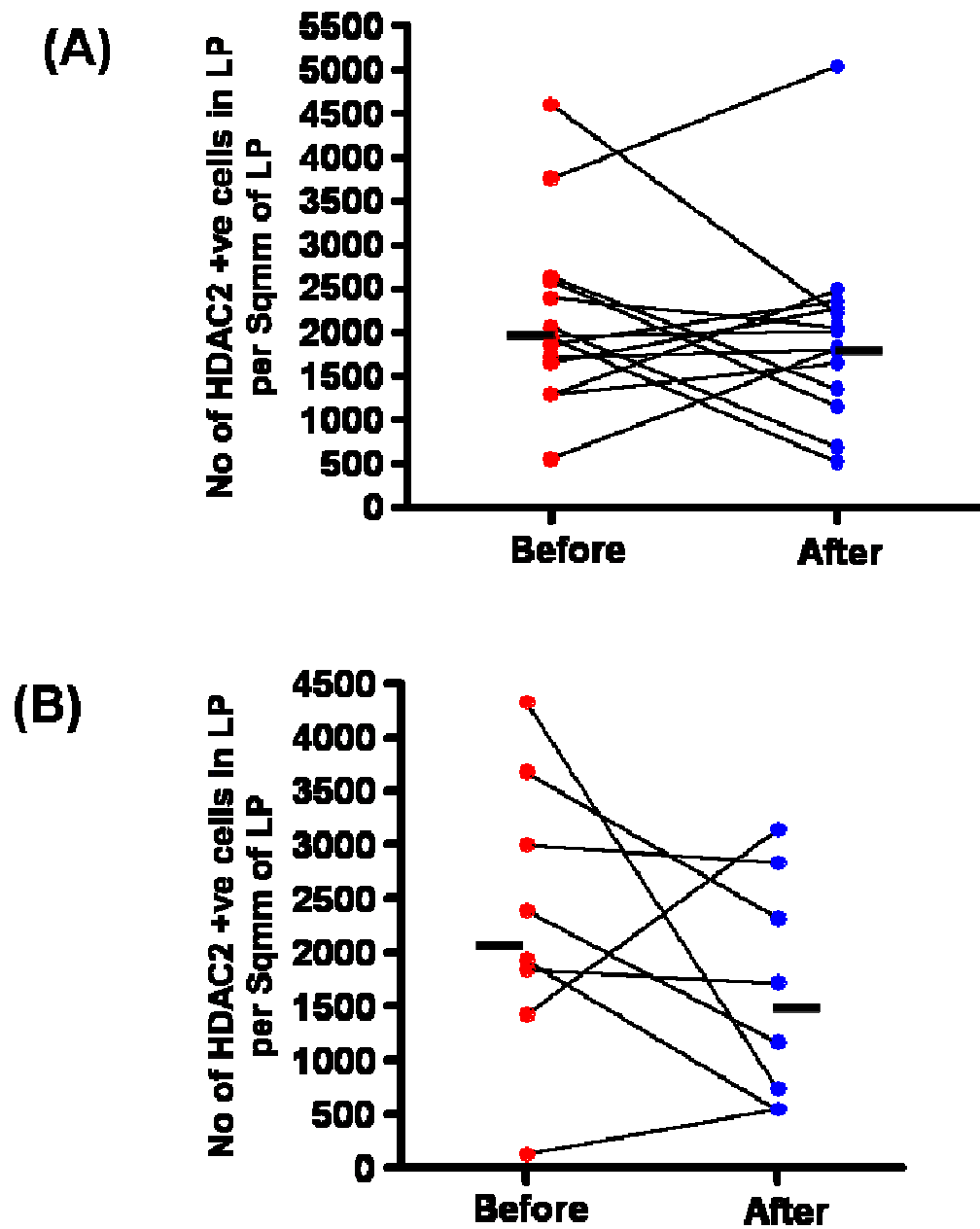


Figure 7.8: Number of HDAC2 positive cells in the lamina propria per Sqmm of lamina propria on treatment with (A) fluticasone propionate and (B) placebo: the horizontal bars represent the mediums. There was no significant difference comparing before and after treatments.

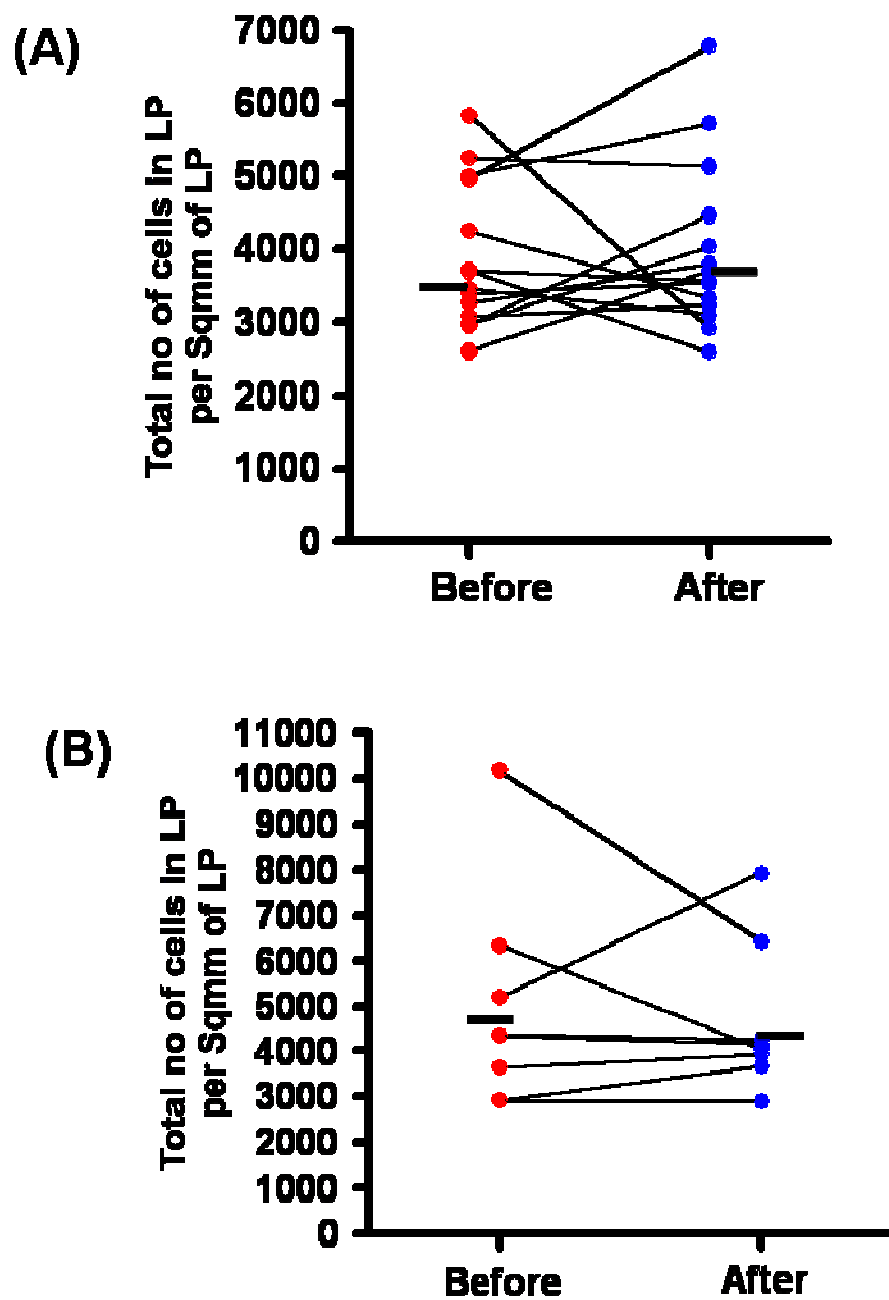


Figure 7.9: The total number of cells in the lamina propria per Sqmm of lamina propria on treatment with (A) fluticasone propionate and (B) placebo: the horizontal bars represent the medians. There was no significant difference between before and after treatments.

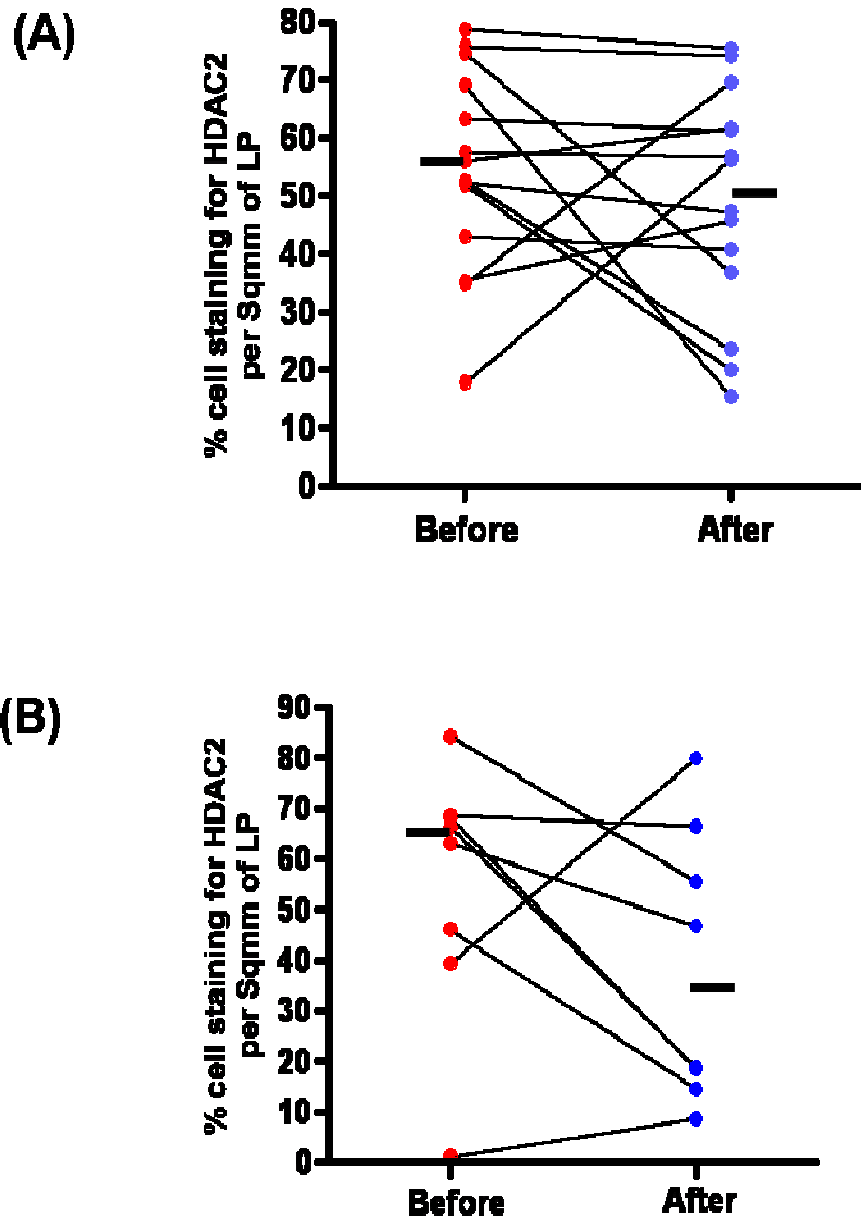


Figure 7.10: Percentage cell staining for HDAC2 in the lamina propria per Sqmm of lamina propria; **(A)** with fluticasone propionate and **(B)** with placebo: the horizontal bars represent the mediums. There was no significant difference comparing before and after treatments.

7.6 Discussion

To the best of my knowledge, this is the first detailed biopsy immunostaining study looking at the status of HDAC2 expression in COPD and its potential reversibility with ICS or/and smoking cessation (albeit the latter in a cross-sectional study only at this stage).

The airway epithelium showed strong positivity for HDAC2 staining/expression but, interestingly this was not different between the groups. Although COPD current smokers showed a slight decrease in HDAC2 staining in the epithelium (as percentage of HDAC2 area of staining in the epithelial area measured), this was not statistically significant when compared to normal controls. However the difference between groups, even if real, hardly suggests that this is a major effect and HDAC2 is more variable between individuals than groups. The fact that HDAC2 expression in the epithelium is well preserved in smokers (even if little less in current smokers COPD subjects) suggest that ICS may still be effective in this tissue compartment, as the CS-GCR complex should be able to access its transcription sites as CS-sensitive genes.

In the lamina propria the data were very striking. I found that there was a decrease in the total number of cells in the lamina propria in COPD current smokers compared to normal controls. This total cell change was strongly related to smoking history and is an effect not previously described. Normal lung function smokers also showed a slight decrease in total cellularity whilst the COPD-ES group showed a normalisation in total cellularity.

In the COPD subjects the plot clearly indicates that smoking is affecting the total cellularity of the lamina propria. Notably, if I had used molecular techniques as used by the Barnes group, etc I would have come to a false conclusion as I would not be aware that there is a dramatic change in total cellularity in the lamina propria, largely causing the apparent down regulation of HDAC2 mRNA and decreased protein content. For example (as explained in Chapter 2 as well), Ito et al (Ito et al., 2001), showed by using western blotting that HDAC2 protein content was reduced in bronchial biopsies from normal lung function smokers compared to normal controls,

but looking now at the images they actually published in their paper (but which they did not comment on), it is quite clear that total cellularity of the lamina propria in their normal lung smokers was also substantially less than in their normal controls. But, as stated above, their analysis did not take into account the overall decrease in cellularity and therefore the consequential total protein available. ICS made no difference to the total number of cells in lamina propria.

Our data for HDAC2 positive cells in the lamina propria was quite interesting as well. It suggested that HDAC2 positive cells decrease in the lamina propria of current smokers with COPD compared to normal controls and on the other hand there was an increase in HDAC2 positive cells in the lamina propria of COPD ex-smokers compared to COPD current smokers; essentially returning to normal. Data on normal lung function smokers was quite remarkable; paradoxically they had significantly higher numbers of HDAC2 positive cells in the lamina propria. This suggests that smoking is actually stimulating anti-inflammatory HDAC2 levels, although ICS made no difference to HDAC2 positive cells in lamina propria.

The data on percentage cell staining for HDAC2 further confirmed the above data on normal lung function smokers that the percentage HDAC2 staining was also significantly high in normal lung function smokers compared to other groups, but COPD-CS, COPD-ES and NC were quite similar to each other. This strongly confirms at least the possibility of smoking stimulating anti-inflammatory HDAC2 expression in chronic smokers. However, the decrease observed in COPD in relation to HDAC2 does seem most likely due to changes in cellularity, and not HDAC2 itself. ICS made no difference to percentage cell HDAC2 staining.

Taken together, these data suggest that smoking itself stimulates the HDAC2 expression while COPD does not seem to be affecting the HDAC2 status itself, but the apparent decrease in HDAC2 observed in COPD current smokers is due to confounding by changes in total cellularity, which itself is strongly negatively related to smoking history. Quitting does seem to have a potential for upregulating HDAC2 at a cell level as shown by an increase in number of cells positive for HDAC2 in the lamina propria of COPD ex-smokers and further supported by slight increase in percentage cells staining in COPD ex-smokers, although this was not statistically

significant when compared to COPD current smokers (type 2 error). Most of the effect seems related to change in total cell numbers. However, we need long term smoking cessation studies with larger cohorts to confirm and tease out these findings.

More precisely, the key finding is the difference in HDAC2 expression in normal lung function smokers and decrease in cells in COPD current smokers. It probably suggests that smoking stimulates anti-inflammatory HDAC2 in non-COPD smokers, but in those who develop COPD then HDAC2 goes down but mainly due to decrease in cellularity of lamina propria and not HDAC2 expression itself. Moreover the status of HDAC2 is not different in the epithelium in either smokers or COPD. So essentially smoking is stimulating HDAC2, but decreasing the total lamina propria cellularity in COPD current smokers. It is possible that this difference between smokers with and without COPD may be important in the aetiology of COPD ie if in an individual smoking decreases cells more than stimulating anti-inflammatory HDAC2, then COPD might ensue.

I cannot say at this stage which particular cell type(s) is decreased in the lamina propria in smokers. For more information I would need double staining studies for different cells in the lamina propria ie HDAC2 plus specific cell type marker. It is also suggested that in COPD there is increased apoptosis but this is controversial and is not clearly established. However, increased oxidative stress produced by tobacco smoke and protease–antiprotease imbalance, and also genetic susceptibility, may contribute to increased apoptosis in COPD airways (Park, Ryter, & Choi, 2007; Plataki et al., 2006). In another parallel study in our group (Soltani A, 2009) we found that there is a significant reduction in total number of vessels in the lamina propria in smoking/COPD, so decreased vascular supply might be contributing to decreased cellularity in COPD airways, but we need further studies to confirm that.

In summary, our data suggest that HDAC2 expression is increased in normal smokers but reduced in current smokers with COPD, though the latter seems largely due to confounding by changes in cellularity in the lamina propria but not in HDAC2 itself. Quitting does seem to have a real effect on up-regulating HDAC2 at a cell level, but it is not affected by ICS. We need comprehensive immunohistochemical studies to fully understand cellular changes in the lamina propria and prospective

long term smoking cessation studies with larger cohorts to confirm these findings. Molecular methods must take such changes in the cellular environment into account and cannot be taken on simple face value; indeed the current published data is likely to be misleading because this was not taken into account.

Chapter 8

Summary and General Discussion

8.1 Overview

COPD is a multifaceted disease, with underlying pathophysiology not clearly understood. It is pathologically and physiologically complex with a number of overlapping and interacting components. However, physiologically it has been most defined by irreversible airway obstruction and pathologically by chronic airway inflammation leading to airway wall remodelling and thickening. Symptoms may include impaired exercise capacity and dyspnoea and if associated with chronic bronchitis, patient will also have chronic productive cough. Tobacco smoking is considered as the main aetiological factor associated with the disease at least in western countries, but why only a minority of smokers develop COPD is not clearly understood (details of this discussion are given in Chapter 2).

My thesis reports very novel findings of Rbm fragmentation which I propose as the “hallmark” of EMT in COPD, not described in the literature before. This will add significantly to the body of literature on airway remodelling. My finding will also add to the corpus of knowledge on the status of anti-inflammatory HDAC2 in COPD. The thesis is divided into five main chapters of results. The first chapter involved a preliminary study into EMT in COPD current smokers followed by a more definitive cross-sectional study into EMT markers in Chapter 4. In Chapter 5, I further confirmed EMT by taking into account potential confounding by inflammatory and immune cells. Chapter 6 was a longitudinal analysis into the effects for potentially normalising Rbm fragmentation. Chapter 7 involved a cross-sectional and longitudinal analysis looking at the status of HDAC2 expression in COPD and its potential modulation with ICS and/or smoking cessation.

8.1.1 Preliminary observations

In the preliminary observations described in Chapter 1, I found that the Rbm in COPD current smokers has highly fragmented with elongated spaces and cracks (termed “clefts”) with cells within them. The literature strongly suggested that this could be a hallmark of EMT (Acloque et al., 2009; R. Kalluri, 2009; Raghu Kalluri & Neilson, 2003; R. Kalluri & Weinberg, 2009; Zeisberg & Neilson, 2009). Following this initial exciting finding I undertook a preliminary study using the classic EMT markers most favored in literature (S100A4, MMP-9 and EGFR) applied to sections from bronchial biopsies obtained from COPD current smokers, as I thought it likely that EMT would be most active in these individuals.

This preliminary study demonstrated that the Rbm in subjects with COPD who are current smokers is indeed consistently highly fragmented and that this appeared to be associated with cells expressing the proteolytic enzyme MMP-9. Epithelial expression of EGFR suggested that epithelial cells may be primed for migration, and increased expression of the fibroblast marker S100A4 suggested phenotypic transition to a mesenchymal cell type. These preliminary data suggested that EMT could be active in COPD pathology. Thus, I designed a comprehensive cross-sectional study into the potential for active EMT in COPD, asking whether it is specific to COPD and/or smoking or both, and used material from a longitudinal therapeutic study to see if ICS has any potential for reducing Rbm fragmentation (which is a central feature of EMT).

As a part of my thesis, I also decided to study the status of HDAC2 in COPD. It is suggested that expression and activity of HDAC2 is decreased in COPD due to increased oxidative stress, secondary to airway inflammation, it has also been suggested this may induce (relative) resistance to inhaled corticosteroid therapy in COPD/smokers but there is little confirmatory data on this, especially outside of the Peter Barnes group in London, which is highly committed to this theory. In fact, COPD is responsive to ICS though relatively less clinically, compared to asthma, but this is largely ignored in this conceptual model. Moreover, the methodology used by the Barnes group is questionable and largely dependent on molecular RNA quantitation and protein analysis which could not take into account relative

differences in cellular profiles in airway tissues in different disease and control groups, where biases could arise due to differences in total and differential cellularity. For the protein assays Barnes team attempted to normalise biopsy-extracted proteins against bovine serum albumin (BSA) and mRNA RT-PCR assays with a so called house-keeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and beta-actin, but it is not clear to me, if this is an adequate way to allow for a change in the cell sub population of interest, for example inflammatory cells in the lamina propria. These house-keeping genes can cause confounding (Glare, Divjak, Bailey, & Walters, 2002), but they would also reflect the more global environment of the airway with all its complexity. Therefore, the second aspect of my thesis involved a comprehensive immunohistochemical cross-sectional and longitudinal analysis investigating the status of HDAC2 expression in COPD and asking whether smoking cessation and/or ICS therapy have any potential to raise HDAC2 airway levels in COPD.

8.1.2 Rbm fragmentation and potential EMT in smoking and COPD

This study as introduced above and described in detail in Chapter 4 and Chapter 5, was a comprehensive cross-sectional analysis into the potential for active EMT in COPD, whether it is specific to COPD and/or smoking or both. In this study I found that Rbm is highly fragmented in both smokers and COPD patients, but especially in current smokers with COPD Rbm fragmentation positively correlated with smoking history in the smoking COPD subjects.

Markers of EMT were evident in bronchial biopsies from smokers and COPD patients. Dual immunostaining for S100A4 and vimentin in the basal epithelium and Rbm further strengthened the likelihood that these cells are undergoing transition to a mesenchymal phenotype. COPD ex-smokers were more variable but mostly quite similar to COPD. For S100A4 cells in the Rbm, there were significant more positive cells in current smoking COPD than both other comparator groups (COPD-ES and NS).

The only significant relationship with degree of physiological fixed airflow obstruction was with S100A4 cells in the basal layer of the epithelium in the current

smoking COPD group. These data taken together suggested that the remodelling changes are more exaggerated in established COPD as might be expected, especially in COPD with active current smoking, and that the changes we describe and attribute to likely EMT, may well be of pathophysiological relevance.

The positive findings for EMT markers in smokers with normal lung function, compared to normal non-smokers, suggest that smoking itself has the potential to initiate EMT. Furthermore, our data in the ex-smoker COPD group suggest that once these changes are initiated they may be irreversible. However, the observations that there fewer cells expressing S100A4 in ex-smokers in COPD ex-smokers in the Rbm but no difference in basal epithelial cell S100A4 staining, may suggest that migration from the epithelium across the Rbm decreases following smoking cessation, to confirm this I need long term smoking cessation.

In summary, the main message of the study was that smoking has potential to initiate EMT, including Rbm fragmentation, but that this is most marked in currently smoking COPD. It also suggested that smoking cessation has the potential to reverse the process to some extent.

The potential criticism of the study is that the cells in the Rbm positive for S100A4 could be infiltrating inflammatory or immune cells rather than epithelial cells undergoing transition. So, to confirm the epithelial origin of these cells in the Rbm and to address the potential for our data to be confounded by infiltrating inflammatory or immune cells, I undertook double immunostaining with epithelial and mesenchymal markers and also specific comparative staining for infiltrating inflammatory or immune cells in matched slides.

8.1.3 Confirming EMT

In Chapter 5, I followed on from Chapter 4 and undertook double immunostaining with epithelial and mesenchymal markers and also specific comparative staining for infiltrating inflammatory or immune cells in matched slides. The comparative analysis demonstrated that these cells in the Rbm are of epithelial origin (by double staining for cytokeratin) and they stained with a mesenchymal marker (vimentin)

suggesting phenotype change; and they are not infiltrating macrophages or fibroblasts, nor CD4+/CD8+ T lymphocytes or B-cells or dendritic cells. This further confirmed EMT and also demonstrated that my data is not confounded by infiltrating inflammatory or immune cells.

8.1.4 Effect of ICS on Rbm fragmentation: potential for the amelioration of EMT

In the previous chapters (as shown above), I concluded that EMT is likely to be an active process in COPD. A key question is whether ICS have any potential for amelioration or even reversal of EMT in COPD. To answer this question I performed a longitudinal analysis, described in Chapter 6. This analysis revealed that fluticasone propionate has potential for normalising EMT in COPD using quantitation of Rbm fragmentation as the illustrative and “classic” manifestation of the process. The study was limited by the fact that I haven’t yet undertaken comprehensive longitudinal analysis with direct cellular markers of EMT as described in Chapter 4, but this was due to the time limits I had with my PhD submission. However, I need now complement this work with a wider range of markers of EMT to confirm and extend these preliminary findings.

8.1.5 HDAC2 in COPD

As part of my thesis I also looked at the status of HDAC2 expression in COPD and smoking using immunohistochemistry and its potential modulation with ICS and/or smoking cessation, described in detail in Chapter 7. This was the first comprehensive airway biopsy study looking at the status of HDAC2 in COPD and smoking.

The data regarding HDAC2 was very striking and quite different to what others have reported using molecular techniques. Interestingly, HDAC2 expression was not different in the epithelium between the groups. The fact that HDAC2 expression in the epithelium is well preserved in smokers (even if little less in current smokers COPD subjects) suggest that ICS could still be effective in this tissue compartment, as the CS-GCR complex should be able to access its transcription sites on CS-sensitive genes.

I found that smoking has the potential to stimulate total HDAC2 expression, potentially increasing the anti-inflammatory environment in the airway wall, as shown in normal lung function smokers. This does seem to be through a direct action on HDAC2 status itself, as the total numbers of cells in lamina propria were lower than normal in number while total HDAC2 positive cells were higher in number (significantly) and percentage HDAC2 positive cells therefore were also increased (significantly). In contrast, a decrease in HDAC2 was observed in COPD current smokers (described in terms of reduction in HDAC2 positive cells in the lamina propria), but this was largely due to confounding by a decrease in total lamina propria cellularity compared to normal, this lowering in cell number was strongly negatively related to smoking history. The percentage of HDAC2 positive cells in COPD current smokers was down slightly compared to normal but not significantly so. There was a significant difference in HDAC2 percentage cell staining between smokers with COPD (lower than normal) and physiologically normal smokers (higher than normal, as above)

Quitting smoking did seem to have a potential for up-regulating HDAC2 at a cell level as shown by an increase in number of cells staining positive for HDAC2 in the lamina propria of COPD ex-smokers and also a slight increase in the percentage cells staining. However, we need long term smoking cessation studies with a larger cohort/size to confirm these findings.

A key finding, I believe, is the difference in HDAC2 expression between normal lung function smokers and COPD current smokers. This may reflect a fundamental difference between individuals in response to cigarette smoke and through this vulnerability to developing COPD ie if in an individual smoker decreases cells more than stimulating anti-inflammatory HDAC2, then COPD might ensue. Moreover the status of HDAC2 is not different from normal in the epithelium in either smokers or in COPD. ICS made no difference to HDAC2 status in COPD, with little likelihood of a type-2 error in this finding.

We now need comprehensive immunohistochemical studies to fully understand smoking-related cellular changes in the lamina propria especially in COPD, and prospective long term smoking cessation studies with large cohorts to confirm these

findings. Molecular methods must take such changes in the cellular environment into account and cannot be taken on simple face value; indeed the current published data is likely to be misleading because this may not have been taken adequately into account.

8.2 Final Conclusions

My findings reported in this thesis have confirmed that EMT is an active process in COPD and is most likely initiated by smoking, but most exaggerated in active smoking COPD. Smoking cessation and ICS both have the potential to reverse the process of EMT. This finding of EMT in COPD adds to understanding about the remodelling process in COPD and to knowledge on pathophysiology of COPD. It may also help to understand why lung cancer is so common in smokers with COPD, and indeed why it is so aggressive, invasive and fatal in over 80% of cases. Observations regarding the status of HDAC2 in COPD reported in this thesis are novel and will rebalance the bias currently in the literature. The data confirm that HDAC2 expression is stimulated in physiologically normal smokers, but down-regulated in smoking COPD individuals but the latter effect is due to smoking-related decrease in lamina propria cellularity.

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Response to examiners

Response to examiner 1

List of figures and tables are missing.

I agree with the examiner's comment and a list of figures and tables is now provided in the thesis.

Chapter 1

Primary comment

The link between COPD and lung cancer should be discussed, given the known correlation between TGF-induced EMT and cancer metastasis and the susceptibility of COPD subjects to lung cancer. Were all of the patients in the preliminary study cancer-free?

All patients were cancer free clinically, radiologically and bronchoscopically at the time of the studies, and we are not aware of any subsequent change in status. I acknowledge the examiner's comment that there was little discussion of the potential link between COPD and lung cancer, in an attempt to avoid being over-speculative. But it is a reasonable point, and I have included some discussion on Chapter 1 page 9 and 10.

Minor comments

Re EMT in lung transplantation and investigation of EMT markers on epithelial cells: Borthwick is quoted but the previous reports by Ward 2005 and Hodge 2009 should also be referenced here.

In the introduction from Chapter 1, Ward 2005 was quoted as a reference 5 times and in the discussion 4 times. I agree with the examiner's comment that reference from Hodge 2009 was not quoted because the study reported by Hodge and et al was mainly an in-vitro cell culture study, but was quoted in Chapter 2 (literature review), pages 77 and 78 of the thesis.

Chapter 2

Primary comments

Given the important role played by HDAC-I in TGF induced EMT (Lei rnt J Biochem Cell BioI 2010) and applicant's focus on EMT in COPD; it is puzzling why HDAC-I was not also investigated.

I agree with the examiner's comment that HDAC1 may play an important role in TGF-induced EMT, but the aim of the study was to first confirm EMT in COPD (as this is the first study in the literature reporting active EMT in COPD), which in itself was a very comprehensive study. Now that we have confirmed that EMT is active in COPD, the next aim of this programme will be to investigate the driving mechanisms behind EMT in COPD and HDAC1 would be a very reasonable target. We already have NHMRC grants pending to take this work further. I have not added any

comment on HDAC1 to the thesis, as it is not immediately relevant to my work described in the thesis.

P62, Figure 2.19. This is of very poor quality in my version and should be re-done.

I thank the examiner for this and the Figure 2.19 is now re-done as suggested (Chapter 1 page 63).

Minor comments

Page 17. What are figures in A\$.

Financial figures are now given in A\$ on page 18.

Chapter 3

Primary comments

3.1 Introduction line 15. Was this really a longitudinal study (that involves repeated observations of the same items over long periods of time)? Or a double-blind, placebo controlled randomised trial?

It was indeed a double blind randomised controlled trial comparing fluticasone propionate (0.5 mg/twice daily) with placebo for 6 months as explained on page number 101. It was also, of course 'longitudinal', but I take the point being made and have altered the title of the chapter accordingly (page number 177).

I assume that the pharmaceutical company supported this study? Was their approval necessary or obtained?

The original basic clinical and bronchoscopic study was supported by GSK. It covered the first phase of analysis which involving clinical and inflammatory outcomes. This work has been published (Reid et al., 2008), and formed the core of the thesis presented successfully in 2006 by Dr. Yudong Wen titled "smoking-related airway inflammation and corticosteroid responsiveness in smoking related COPD". That funding ceased in 2004, but was a completely untied grant. Although it was always intended to go on and study airway remodelling in the context of the biopsy samples obtained, the details of this only recently emerged with my pilot work and were not at all under the direct auspices of GSK. No permission for the analysis was needed or sought from them.

2.3.7 were the Mabs titrated for optimal staining?

Yes, monoclonal antibodies were titrated for optimal staining. Details are given in Chapter 3, pages 104 and 105.

3.3 The use of lignocaine should be discussed given its negative effects on epithelial cell viability (Kelsen SO Am J Respir Cell Mol Biol. 1992 (1):66). This is potentially increased in COPD where there is fragility of airway surface epithelium (Jeffery PK 2001 Novartis Found Symp. 2001; 234).

This may be a danger for some confounding but really this would only be the case with living cells e.g. BAL samples, rather than with fixed tissue. Our group some years ago studied this phenomenon in detail (Duddridge, Kelly, Ward, Hendrick, & Walters, 1990). It is extremely unlikely that the changes we have seen are related to lignocaine, both on the basis of this work, but also because the control subjects were

treated in a exactly same way as those with COPD and, as explained in the thesis (Chapter 1), we have never found such changes in the Rbm in asthma subjects studied over the years, although again treated in the same way.

A demographic table for the subjects used in the main study is required here. In the demographic table provided for the PILOT study, there was a large difference in median ages for controls vs COPD patients: 47 vs 61. Even more concerning were the inclusion of very young subjects in the control group for the PILOT study (age 20). Can you be sure that there were no effects of increasing age on your findings?

A demographic table for the subjects is now provided in Chapter 3, page 103 as suggested by the examiner. I acknowledge the examiner's comment that some of the controls subjects were of relatively young age for a study of COPD, but as explained in the thesis (pages 106 and 135) linear regression analysis was undertaken and age was not related to the findings we observed in the COPD or smoking populations.

3.2.2 Do you consider 6 months cessation of smoking to be enough for reversal of the Rbm changes?

The inclusion criteria stipulated that COPD ex-smokers had to have at least six months of smoking cessation , but this really was a `theoretical` minimum and as a group they had considerably more time from quitting than this (usually years). In the event, we found that S100A4 positive cells in the Rbm were decreased in these COPD ex-smokers and there was no indication that this was related to time since smoking cessation. In the near future I/we plan to analyse a specific smoking cessation study for which we have serial biopsies from 15 individuals before and after they had ceased smoking for 3 months and 12 months .This is supported by our current NHMRC grant and should give some indication to the time scale of these changes.

3.5 Presentation of the immunostaining techniques pp 109-131 is tedious and could be condensed. Details of manufacturers: city/state or city/state/country are missing or incomplete.

Considering the complex nature of the processes being investigated for the first time in COPD, details of the stains done seemed to me very important, especially if anyone else wishes to replicate the work. I would prefer for this reason to leave the section unchanged. As requested, I have updated the manufactures` details and sources of the antibodies in the thesis (Chapter 3, pages 104 and 105).

Chapter 4 **Primary comments**

The inclusion criteria states 'over 18' in the controls and '40-70 years' for the COPD group (described in 3.2.2) compared to COPD subjects aged '40-70y'. However, in 4.3 ' Results', COPD subjects of 78 years of age are included. These subjects should be removed and stats redone.

I thank the examiner for picking up this inconsistency, which reflects a change in recruitment policy after the original protocol was designed; and inadvertently it was this I was quoting. I should have quoted the operative inclusion criterion for age, which was for COPD subjects over 40 years old but with no upper limit as long as clinically fit enough for the procedures (page 103). This is now altered in the thesis.

Chapter 5

Primary comments

Re low numbers of-cells in biopsies. There appears to be surprisingly few T-cells in the biopsies. Do you think this could be related to severity of the COPD subjects as reported by Di Stefano 2001; 31:893?

The data referred to are part of a comparison of cells positive for S100A4 in the basal epithelium and in the Rbm versus T-cells and other immune cells within these structures. This was specifically done to show that cells in the basal epithelium and Rbm are of epithelial origin and that the data are not confounded by infiltrating inflammatory and immune cells. One would not expect to see many such cells normally in these areas.

Chapter 6

Primary comments

Page 168 " it is likely that in COPD the epithelium may get damaged by cigarette smoke". There have been several studies that have very convincingly shown that cigarette smoke does damage the epithelium and/or cause remodelling; these should be discussed (e.g., Pera 2010, Zhao 2009).

The specific reference has been added to the introduction mentioned on page 169; further details on epithelial damage and airway remodelling are given in detail in Chapter 2.

6.5 Discussion, last para. How exactly will you do a comprehensive longitudinal follow up study" Won't the invasive nature of the airway biopsy procedure preclude repeated observations over long periods of time?

As already discussed, in the near future we plan to analyse a specific smoking cessation study for which we already have serial biopsies collected.

What are the potential mechanisms for the effects of ICS on Rbm fragmentation- direct effect or via effects on inflammatory mediators or injury? How will this be assessed in future studies?

The exact mechanisms for how ICS work in COPD are not very clear, but potential molecular mechanisms involved are described in the Chapter 2. It is very hard to say anything about the exact mechanisms involved in normalising the Rbm at this stage as the findings on Rbm fragmentation are so new. However, we already have plans, and a funding grant submitted, to investigate the role of RAGE (Receptor for advanced glycation end-products) as a driver of these `amplification` remodelling changes in the context of chronic airway inflammation in COPD. We will then be able to see if this is the level of control. This itself may be under the influence of VEGF or TGF- β , and we wish to dissect these factors out from each other using the intervention studies we have done.

Chapter 7.

Primary comments

P 177 it is rather harsh to claim that the methodology used in the Barnes study were "rather questionable". Rather they were not comprehensive enough to identify the changes in total cellularity in the lamina propria.

The examiner's phraseology is probably more polite and reasonable, and I have changed the use of words in Chapter 7, page 178.

Minor comment

7.3.5 How was normality assessed?

Normality was tested using the Kolmogorov Smirnov & Shapiro Wilk test (Chapter 7 page 180).

Discussion

Primary comments

PI99 Para 2. How do you anticipate doing the “long term smoking cessation” study? Would a murine smoking model be appropriate?

As explained above, we already have serial biopsies from a smoking cessation study involving 15 individuals over 12 months. Since we have human tissue available, we do not need to revert to animal models.

In future studies, a discussion of potential future studies investigating the signaling pathways extensively discussed in pp 64-73 would be appreciated.

As already mentioned we are now intending to go on and investigate such mechanisms. We will focus on RAGE and TGF- β 1 systems and their second messengers. To assess the drivers of airway inflammation and remodelling, we will immunostain airway biopsies for expression of RAGE in epithelium, endothelium and inflammatory cells. We find that TGF β ₁ is highly and indiscriminately expressed in airway biopsies from all groups, so for this core mediator we will use quantitative PCR (qPCR) on extracted biopsy mRNA for the growth factor itself and use both immunostaining and molecular quantitation for its strategic transcription factors SMAD-2,-3 and -7. We will also stain for the downstream major pathogenic trigger TWIST and quantitate it by molecular means; and will also perform these measures on matched cell pellets (mainly macrophages) from BAL (bronchoalveolar lavage; essentially small airway washings) which have been stored for each individual.

However, I feel that these studies are beyond the scope of my thesis as planned and written. The work is already rather long, as noted by the examiner, and to do adequate justice to these complex issues would require a whole new set of areas to discuss at length, and properly reference etc (there is now a substantial literature on this) if we were to go down this avenue, which I would prefer to avoid.

Typographical errors

All the specific typing errors picked up by the examiner have been corrected.

Response to examiner 2

Minor points

Figure 1.1 Assume the asthmatic was a non-smoker

I acknowledge examiner's comment. This asthma subject was a non-smoker and the figure legend is changed accordingly (Chapter 1, page 4).

In the study reported in chapter 1.2.1 and associated table, clarity on how the asthma phenotype was excluded in the COPD patients and excluded from the controls would help ensure no overlapping phenotyping effects. The age was different and no discussion about the effects of age on Rbm clefts/fragmentation was mentioned.

As explained in Chapter 3, page 101 & 102, diagnosis were made by a respiratory physician clinically, physiologically and ultimately pathologically. Subjects with a history suggestive of asthma, which included symptoms in childhood, related atopic disorders, eczema or hay fever, substantial day-to-day variability or prominent nocturnal symptoms, or a history of wheeze rather than progressive breathlessness were excluded. Any previous medical diagnosis of asthma was also an exclusion. None of our COPD subjects had an airway histological picture that looked like asthma.

I acknowledge the examiner's comment that some of the control subjects were of young age, there was a wide spectrum of age in the control group and in reality the differences between smokers/COPD did not depend on the mean age difference. As explained in the thesis, linear regression analysis was undertaken within the COPD group and age was not related to the findings.

In chapter 3.2.1 exclusion criteria including other co-existing respiratory disorders was loosely described ie was CT routinely used to exclude fibrosis and bronchiectasis as these are often incidentally found to co-exist in COPD.

CT scanning was not performed, but clinical and physiological examination was done, and subsequently obtained BAL fluid had to be free of bacterial growth/colonisation. Thus, there was no clinical (e.g. crackles on auscultation) or radiological evidence of fibrosis; and no clinical, radiological or microbiological evidence of bronchiectasis.

Discussion in 4.4 should acknowledge that the (a) smoking controls are younger and have lower pack years exposure and that adjustment for this does not effect the findings which and (b) that changes consistent with EMT was tracking with smoking per se and the specific association with COPD and SIOOA4 cells was significantly only after correction for Rbm length but not reflected in the cleft number. (pg 151).

The issue of age variations has already been dealt with above.

I agree that the changes I have concluded are related to EMT are related to smoking per se, but the data also strongly suggest that the changes are especially marked in COPD current smokers. This was extensively discussed in Chapter 4, pages 152-155.

The use of normal controls for comparative purposes in the chapter 6 study is problematic as they are younger with less smoking history. I; m sure this

researcher would have preferred to have examined changes in biopsies in the same patients before and after treatment as a more rigorous means of comparing effects of steroid vs. placebo on EMT characteristics in equally matched groups. If the placebo group is smaller then any effects may be lost due to under-powering.

The realities of doing intervention studies with bronchoscopic outcome measures include the fact that one needs to optimise the material obtained. Our group has made the point on many previous occasions that the control data is frequently largely wasted; it is there to try and re-assure the reader that no change is spontaneously occurring. In the study included in the thesis there is just no suggestion of a consistent change in the control group, with no realistic chance of a Type-2 statistic error. Further, although the sample size was small in this study, as explained in Chapter 6, I did perform power and sample size calculations for this analysis, which showed that a number of subjects smaller than 15 was adequate to find differences for Rbm fragmentation before and after treatment with an alpha error 5% and beta error 80%. This is quite consistent with previous work from our group published some years ago by Richmond et al (Richmond, Booth, Ward, & Walters, 1996).

I would have liked to have seen a stronger argument that supported the hypothesis that ICS might affect EMT through some mechanism explained in chapter 2.

This issue was dealt with in detail in response to examiner 1.

Typographical errors

All the typing errors mentioned by the examiner have now been corrected in the thesis. I am grateful to the examiner for such a careful and helpful reading of the text.

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